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THE BIOLOGICAL BULLETIN

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ON THE ORIENTATION OF CENTRIOLES IN DIVIDING CELLS, AND ITS SIGNIFICANCE: A NEW CONTRIBUTION TO SPINDLE MECHANICS[1]

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The centriole is one of the most fascinating and baffling of the cell components. Despite the painstaking and inspired work done on this cell organelle during the last quarter of the nineteenth century, its very existence as a real structure was called into question within recent times (Fry, 1929). The idea that the centriole is a fixation artifact, without organic continuity, was refuted brilliantly by E. B. Wilson and his students, and the continuity of the centriole from one cell generation to another is now firmly established, for some materials at least (Wilson, 1930; Sturdivant, 1931; Wilson and Huettner, 1931; Johnson, 1931; Huettner, 1933). The observations on the centriole recorded by the older generation of cytologists, including Flemming, Boveri, Conklin, Lillie, Mathews, van Beneden, Heidenhain, Mead, MacFarland, and many others, were not only substantiated but actually reinforced by this controversy.

Despite all this interest, the actual function of the centriole in fertilization and cell division has remained essentially unelucidated, because of the anomalous situations existing in the *de novo* origin of centrioles during parthenogenesis, in the existence of primary and secondary centers during the normal fertilization of certain eggs, and in the well demonstrated existence of anastral and acentriolar mitosis in many forms, including the higher plants.

The great variation in form and behavior shown by this cell component, and by the centrosome, aster, blepharoplast and other structures related to it, is such, also, as to render controversial many of the hypotheses thus far made about the specific functions of the whole centriole-complex. The centriole itself may occur, in different materials, as a tiny dot-like structure; as a diffuse group of fine particles; as a definitive rod; as a distinct V-shaped body, with or without axial filaments and terminal vesicles; as a ring-shaped circlet; or there may be a complete absence of visible centrioles in certain anastral configurations. These variations present an array of manifestations which are, to say the least, confusing.

[1] Aided by a grant from the National Institutes of Health, RG-5328.

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Recent electron microscope studies (on all too few materials, thus far) have revealed, however, that the centrioles examined to date by this means are cylindrical cage-like bodies composed of 9 rod-like or tubule-like structures embedded in a matrix. The tubules may be double or single. Presumably the dot-like centrioles as seen by visible microscopy are actually very short cylinders, the longer rods longer cylinders, and the V's composed of two attached cylinders. The rings may be very short, very broad cylinders. No electron microscope studies have been made, to my knowledge, of the more specialized types of centrioles or of the finely particulate centriolar areas.

If the majority of centrioles are actually rod-like structures, however, it seemed that it might be profitable to examine one of the few forms in which the rod-shaped centrioles are sufficiently large to be easily distinguishable in fixed material without the use of electron microscopy. One of the purposes of the present paper is to give a more complete description of the remarkable giant first cleavage spindle of the egg of *Polychoerus carmelensis* and its unusual centrioles. Certain comparisons will be made, also, between the resting first cleavage spindle and the non-resting maturation spindles and their centrioles, but a more complete description of the meiotic spindles and their chromosomes will be reserved for a later paper.

Materials and Methods

Polychoerus carmelensis is a small orange-colored marine acoel flatworm from the tidepools of Pescadero Point, Carmel Bay, California, described by Costello and Costello (1938a). It is similar in many respects to *Polychoerus caudatus*, named and described by E. L. Mark (1892) and once common from Great Egg Harbor, New Jersey, to Casco Bay, Maine. Representatives of this genus are fascinating little animals, which as early as 1865 attracted the attention of A. E. Verrill (see his 1892–1893 paper), of Joseph Leidy in 1874 (see Verrill, 1892–1893), of T. H. Morgan in 1890 (personal communication from Ross G. Harrison), and later of E. G. Gardiner (1895, 1898). They have several unique or unusual features, certain of which have been mentioned earlier, in papers (Costello and Costello, 1938b, 1939) or brief abstracts (Costello, 1937, 1946, 1960a, 1960b, 1960c).

Since the egg of *Polychoerus* contains quantities of several types of orange pigment granules, yolk spheres, lipoid droplets, and nuclear remnants of incorporated vitellogenic cells, it is far too opaque for study of the living spindle. Only the dumbbell-shaped outline of the large amphiastral figure can be made out in living material. The primary oöcytes are fertilized and undergo maturation within the body of the hermaphroditic worm, after the worms have reciprocally mated, and there advance to the metaphase of the first cleavage division, at which stage they remain until the eggs are laid and enter sea water. Hence, an ample supply of fixed material, at stages up to and including the resting metaphase, can be obtained by examining the living adults with a hand-lens, and fixing those containing large oöcytes or ova visible through the body wall. The adult worms are quite hardy, and live well in a laboratory aquarium with running sea water, crawling about on the glass sides or wooden walls or on *Ulva* placed in the tank. They may also be kept in fingerbowls if the water is changed at intervals.

A variety of fixatives was used, including Heath's polyclad fixative, Worcester's, Boveri's picro-acetic, Flemming's, Lillie's modification of Meves' fluid, B-15, 20%

formalin, Champy's, Carnoy and Lebrun's, Regaud's #1, and Gilson's fluids. The majority of these were used at three different temperatures: room temperature, warm (about 50° C.) and hot (60–80° C.), with specific temperature records kept in each case. Some egg masses were fixed, also, at various stages after having been laid, and 12 adults were fixed in the act of egg-laying (see Costello and Costello, 1939). All this material was collected and fixed during the author's stay at the Hopkins Marine Station, Pacific Grove, California, during the summers of 1936 and 1937. I am indebted to the late Director, Dr. W. K. Fisher, and his staff for many courtesies.

The fixed animals were individually embedded in hard paraffin, and sectioned serially at 8 or 10 microns. Approximately equal numbers were sectioned transversely, frontally and sagittally. The serial sections were stained by a variety of methods: Heidenhain's iron haematoxylin, Feulgen, Champy-Kull, Benda, Flemming tricolor, safranin, Delafield's haematoxylin, Mallory's triple stain, anilin blue, or crystal violet, and counterstained, in some cases, with eosin, erythrosin, light green, or orange G. Specimens fixed in room temperature Worcester's solution or in Heath's solution and stained with Heidenhain's iron haematoxylin gave preparations of superlative beauty. More than 423 sets of serial sections of the worms and egg masses were prepared by my wife and myself in 1936, 1937 and 1938. Most of these slides were studied by Helen M. Costello and the writer between 1936 and 1940. All the preparations were subsequently re-studied by Dr. Catherine Henley in 1958. This paper would not have been written without this help, and I hereby acknowledge my deep appreciation. My thanks are due, also, to Drs. Sally Hughes-Schrader and Franz Schrader for many stimulating discussions about the *Polychoerus* egg over a period of years, and for reading the manuscript; to Dr. Kenneth W. Cooper for similar conferences; and to Dr. Shinya Inoué for an all-too-brief discussion at Woods Hole during the summer of 1960.

The serial sections were examined at various magnifications (50 to 2400 diameters) and the location (slide number, row number, section number) of each significant feature noted, to serve as a record and in order that any feature might easily be found again. Photomicrographs were taken of maturing eggs, spindles, chromosomes, etc., at magnifications of 300 to 1400 diameters, using apochromatic objectives and compensating eye-pieces. Wash drawings were made by Mrs. Ernest Runyon, to whom I am greatly indebted.

Observations

The giant cleavage spindle of the egg of Polychoerus

The fully grown eggs of *Polychoerus carmelensis,* after fixation, measure 200 to 280 microns in diameter. Considerable shrinkage in certain of the fixatives is indicated by the fact that the cavities in the parenchyma in which the eggs lie are 260 to 320 microns in diameter. In one animal fixed in Champy's fluid, there were no shrinkage spaces around the eggs, and one ovum, just extruded through the body wall, measured 320 microns in diameter. There is every reason to believe that the ova are closely surrounded by the parenchyma when the animals are alive, and that the eggs shrink more than the surrounding tissues.

The resting (metaphase) first cleavage spindle of *Polychoerus* is of the amphiastral type, with the continuous fibers constituting a central spindle (Hermann,

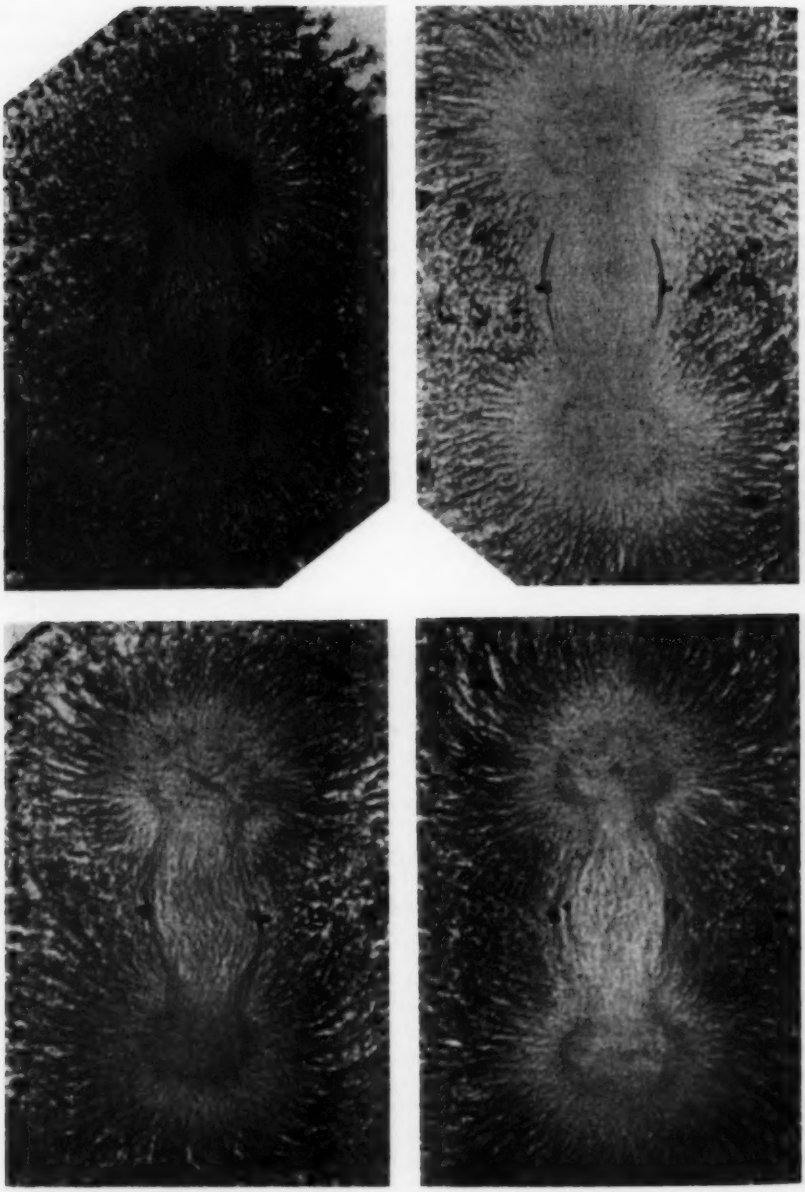


FIGURE 1. Photomicrographs of 10-micron sagittal sections through resting first cleavage metaphase of four eggs of *Polychoerus carmelensis*. Left, upper, fixed in Flemming's; right,

1891) about which the chromosomes are grouped in a ring (Fig. 1). A sheath of mantle fibers lies peripheral to the central spindle, with the chromosomal fibers attached to the kinetochores of the fringing chromosomes. The chromosomal fibers are more fibrous and less granular than the continuous fibers or the astral rays. The chromosomal fibers appear, also, to be compound, consisting of as many as 10 or 12 finer fibrils. The chromosomes are all V- or J-shaped, with median or sub-terminal attachments, and with their arms projecting radially outward from the equatorial plate (Fig. 13). The diploid number of chromosomes, repeatedly counted with ease in a large number of the resting metaphases, is 34. The chromosomes are not exceptionally large, the long arms of the J-shaped chromosomes being 5 or 6 microns in length and the arms of the V-shaped a little less. These chromosomes in the resting metaphase are never double, and have not yet visibly split at this stage.

The fixed spindle at resting metaphase measures up to 120 microns in length [2] between the centers, and a maximum of 65 microns in diameter at the equator. The spindle plus asters often exceeds 160 microns in length. The usual diameter across the equatorial plate is 45 to 50 microns. It should be noted that these dimensions are those of sectioned material; therefore, they do not give the distorted and exaggeratedly spread relationships so often seen in squashed preparations.

In many preparations (especially those fixed in Heath's and heavily stained in iron haematoxylin), a distinct centriole can be seen at each astral center, in most cases in the shape of a rod which may sometimes be straight, but is more often slightly curved, and in a few cases, somewhat twisted. In other eggs, fixed less well, the central region of the aster may show a fine granulation, but no distinct centriole. When curved, the centrioles have their free ends nearer the equatorial plate, the convex portion away from it.

The rod-shaped centrioles of the cleavage metaphase of *Polychoerus* are 4.5 to 5 microns in length by about 0.25 micron in diameter. Examination of large numbers of cleavage spindles sectioned longitudinally indicates that the centrioles at the two ends of a given spindle are never oriented parallel to each other. In fact, while they lie transversely (or, more rarely, slightly obliquely) to the long axis of the spindle, their axes never lie in the same plane but are oriented at right angles to each other and usually at right angles to the spindle axis. Under intermediate magnification, this often results in a figure that gives the superficial appearance of having a rod-shaped centriole at one pole and a small spherical centriole at the other (see Figure 2). Under high magnification (of the light microscope), the spherical centriole can be seen to be the optical section of a vertical rod, at right angles to the horizontal rod-shaped centriole at the other pole. In eggs in which the plane of section did not happen to coincide with the longitudinal axis of the spindle, the same right-angle relationship between the two centrioles of a given spindle can be demonstrated by studying the serial sections. Whatever the angle of orientation of one centriole in relation to the plane of section, the centriole at the

[2] In the ovum fixed in Champy's fluid, the spindle measured 134 microns between the centers and 40 microns in diameter at the equatorial plate. The asters were about 80 microns in diameter.

upper, and both lower fixed in hot Heath's; all stained in Heidenhain's iron haematoxylin. The slender rod-shaped centrioles (0.25 micron in diameter) do not show clearly at this magnification. 550 ×.

other end of the same spindle was found to be approximately at right angles to the first.

Electron microscope studies of centrioles by Burgos and Fawcett (1955), by Porter (1956) and by de Harven and Bernhard (1956) indicate that centrioles are cylinders, consisting of 9 rods in a matrix, as mentioned above. In repeated cases in vertebrate cells, where two centrioles are found close together and therefore probably recently divided, the bundles of rods are oriented at 90° to each other. Here, in this resting spindle of *Polychoerus*, even though the centrioles are at opposite poles, they are also oriented at 90° to each other. Since the centrioles of most vertebrate cells are tiny, measuring about 0.3 to 0.5 micron in length by 0.15

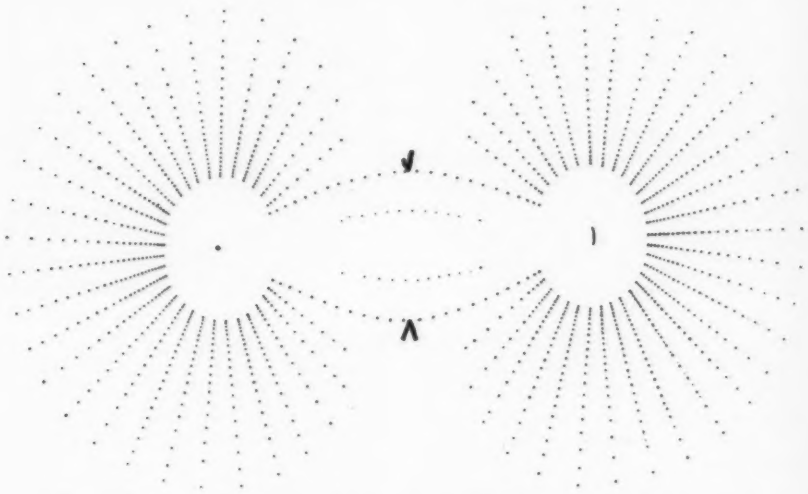


FIGURE 2. Diagram of resting first cleavage metaphase spindle of *Polychoerus*, to show centriolar orientation.

micron in diameter, there is not much chance of discovering the orientation of both of them in a given spindle by light microscopy in any appreciable number of cases. The light microscope does not give a high enough magnification to reveal their orientation. The thin sections used in electron microscopy are unlikely to include both centrioles of a spindle. However, de Robertis, Nowinski and Saez (1960) figure a thin-section electron micrograph (by Bernhard) which includes both poles of a cell in metaphase. Two centrioles are present at each pole, with the recently divided daughter centrioles at each pole at right angles to each other. The distal centriole of each pair is sectioned longitudinally, the proximal centriole transversely, in this particular case. This spindle, however, measured only 5.5 microns (calculated from the stated magnification) between the proximal centers.

Cells of the size of *Polychoerus* eggs are rare and giant spindles are practically unknown throughout the animal kingdom. So far as I have been able to ascertain from the literature, only the spindles of the cleaving egg of the whitefish approach

those of *Polychoerus* in size. In the whitefish, chromosomes extend throughout the equatorial plate, and there is no distinctive central spindle. Consequently, the details of spindle structure and chromosomal arrangement are far less clear.

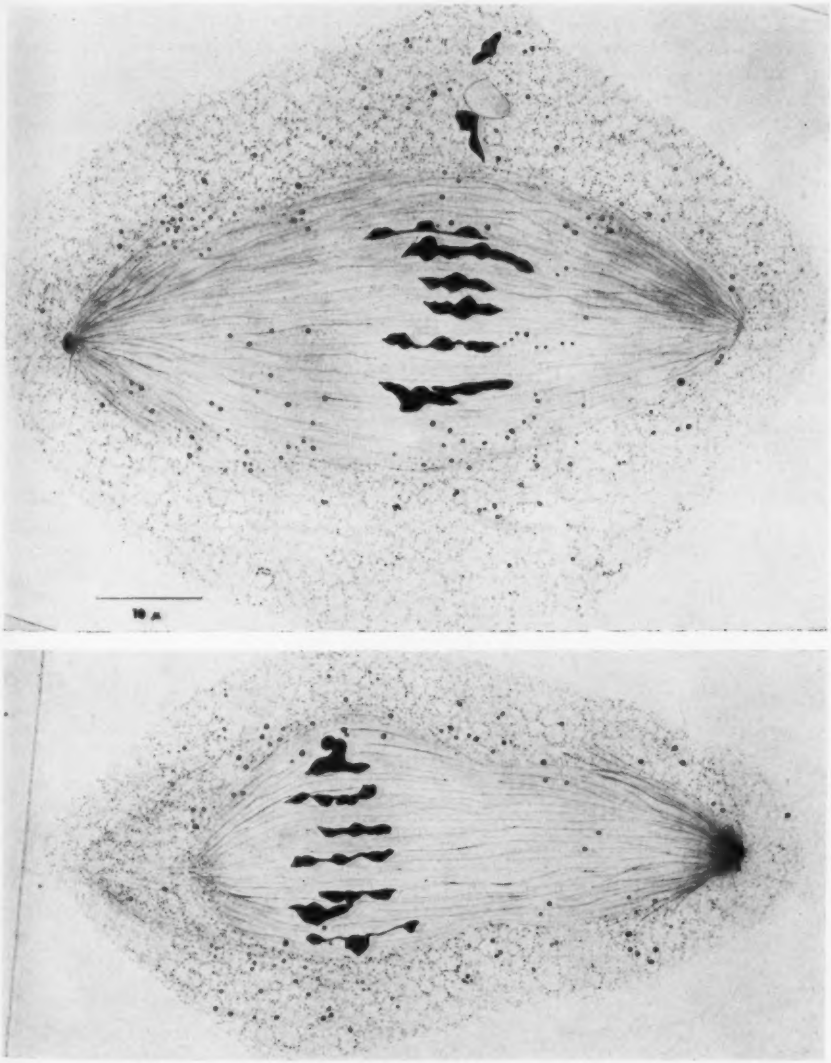


FIGURE 3. Wash drawings, of two adjacent 10-micron sections through metaphase of first maturation spindle of primary oöcyte of *Polychoerus*. Fixation in Worcester's at 60°, stained with Heidenhain's iron haematoxylin and eosin. Figures 3, 4 and 5 were drawn by Laliah Curry Runyon (Mrs. Ernest Runyon).

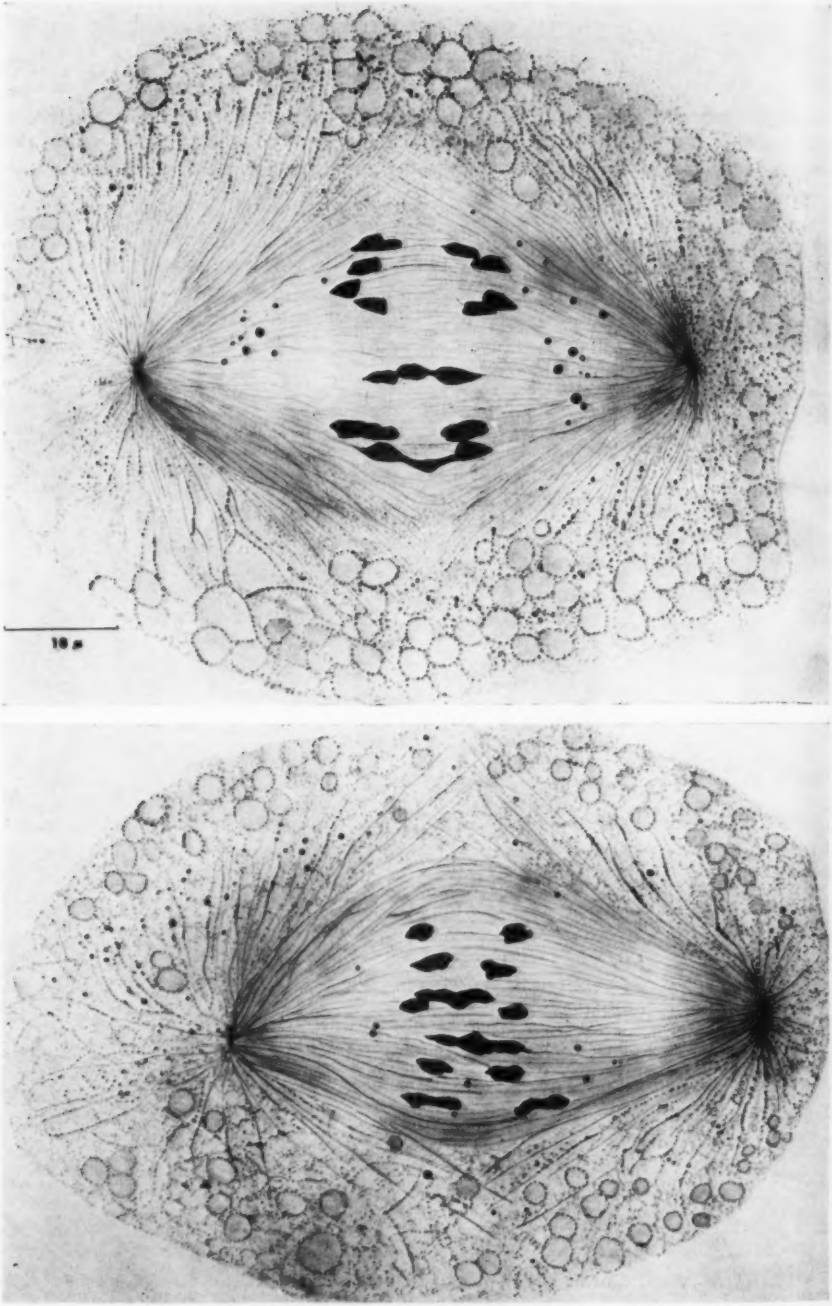


FIGURE 4. Wash drawings of two adjacent sections through first maturation anaphase of primary oöcyte of *Polychoerus*. Fixation: Heath's at room temperature; staining, Heidenhain's iron haematoxylin.

When one considers that one of the most favorable (and largest) cells used for the study of the mitotic spindle, namely, the grasshopper neuroblast, is only 30 microns in diameter, and that most mammalian cells are much smaller than this, the advantages of a form like the *Polychoerus* egg are apparent.

Giant centrioles are, however, not unknown. Cleveland (1935a, 1935b, 1935c, 1938, 1957a, 1957b) has described centrioles (in members of the flagellate genera *Pseudotrichonympha* and *Barbulanympha*) up to 80 microns in length, and in these and in other flagellates, such as *Spirotrichonympha* (Cleveland, 1958), the demonstrated relations between these and the central spindle and chromosomes are rapidly advancing our knowledge of the functions of the centrioles.

The centrioles of the first maturation spindle of the egg of Polychoerus

Since the sectioned material included many animals containing oöcytes fixed while undergoing the maturation divisions, it was imperative to examine these, also, to ascertain the structure and orientation of the centrioles, for comparison with those of the first cleavage spindle. The first maturation spindle of *Polychoerus carmelensis* measures about 75 microns between the centers at metaphase. It is 25 microns in diameter at the equator. Instead of having a central spindle consisting entirely of continuous fibers, there are at least three chromosomes located within the spindle, while the others are more peripherally arranged. This has been observed in transverse sections through the equatorial plate region. The asters of the first maturation division are much less well developed than those of the cleavage spindle, but in the better-fixed material, centrioles are clearly visible at each pole. These centrioles are relatively small, measuring about 1 micron in length by about 0.25 micron in diameter. Some variations in form of the centrioles are found at various stages during the first meiotic division. At the pre-metaphase stretch stage and also at metaphase, centrioles have been found in the shape of curved rods, which may or may not have slightly enlarged ends. These curved rods are oriented with the convex side away from the equatorial plate, the concave side (and free ends) extending toward it. An imaginary line across the free ends of the centriole at one pole is approximately at right angles to the long axis of the spindle, and in almost every case examined was found to be *at right angles to the centriole axis* at the opposite pole of the same spindle. In other spindles at approximately the same or a later metaphase stage, the centrioles were distinctly double at each end of the spindle, consisting of two short rods (0.75 to 1 micron by 0.25 micron), end-to-end or at a slight angle to each other. The apex of the angle was always directed away from the equatorial plate. Obviously these are bivalent centrioles, and they are also oriented at right angles to each other at the opposite poles of each spindle (see Figure 4). There is very little distance (0.1 to 0.2 micron) between the apices of the two rods, and it is possible that some of the centrioles which looked like single curved bodies actually consisted of two parts. They may, however, represent stages immediately preceding duplication of the centrioles, but this requires further detailed study.

There are 17 tetrads on these first meiotic spindles of *Polychoerus carmelensis*. A description of them, and of the stages of breakdown of the germinal vesicle, will be the subject of a later paper. Meanwhile, it may be mentioned that the first meiotic spindle originates as a central spindle of *extranuclear origin*, generated as

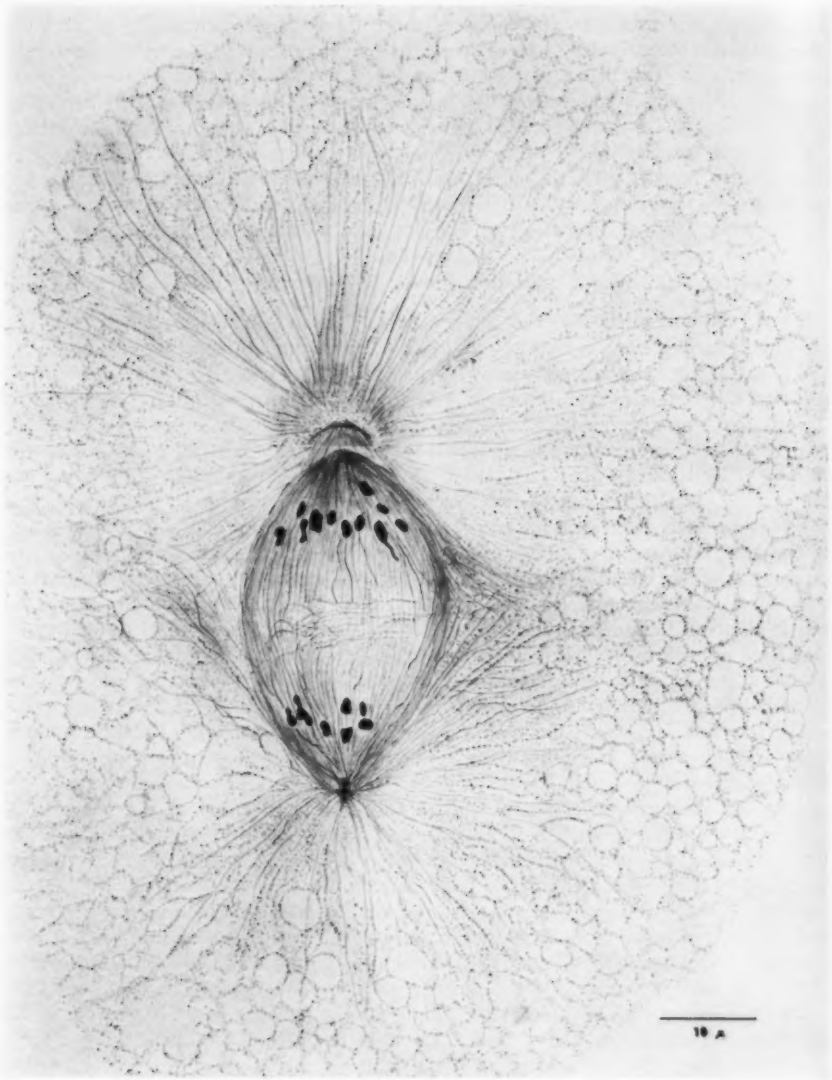


FIGURE 5. Wash drawing of 10-micron section through second maturation anaphase of a secondary oöcyte of *Polychoerus*. Fixation in Worcester's at room temperature; stained in Heidenhain's iron haematoxylin and eosin.

the two primary centrioles and asters move apart and around the oöcyte nucleus, before the wall of the germinal vesicle shows any signs of rupturing. At the same time, in numerous primary oöcytes, smaller secondary centers (with asters), between 5 and 20 in number, appear in the cytoplasm near the wall of the germinal vesicle. These secondary centers may even persist at various points in the cytoplasm, long after the first maturation spindle with its primary centers is fully established. The secondary centers are too tiny for an analysis of their shape. After the rupture of the oöcyte nucleus, the chromosomes become attached to the central spindle and undergo marked pre-metaphase stretch movements.

The centrioles of the second maturation spindle of the egg of Polychoerus

There were not many second meiotic spindles found. These are relatively shorter, broader spindles (38 microns long by 21 microns in diameter at anaphase) than those of the first meiotic division, and contain 17 dyads at metaphase. The astral regions are somewhat better developed than in the first meiotic spindle. The centrioles of these second meiotic spindles are truly remarkable. They are long, thin bodies, curved into a crescentic form. The clearest of these are 5 microns in length and so thin as to be just at the limit of microscopic visibility (*i.e.*, about 0.2 micron). The concave side (and free ends) are directed toward the equatorial plate, the convex side away from it. These resemble the centrioles of the second spermatocyte division of *Nemobius,* as described (his Fig. 39) by Baumgartner (1929), although in the gryllid material the centrioles are straight. At the two opposite ends of the same spindle, the axes of the centrioles are again at right angles to each other (see Fig. 5). There is no indication of duplication in any of these second meiotic centrioles. These appear, then, to be univalent. Presumably because of the orientation of the two centrioles, these second meiotic spindles look much more heteropolar than did the first.

Since the metaphase centrioles at the opposite poles of the first maturation spindle, of the second maturation spindle, and of the first cleavage spindle, even though differing in size, valency, etc., are oriented at right angles to each other, it would seem that this is an inherent property of either the centrioles or the protoplasm of the species.

The details of the origin of the cleavage centers for *Polychoerus carmelensis* have not yet been completely worked out. However, enough stages have been studied (including many examples of the association of the 17 maternal with the 17 paternal chromosome vesicles) to indicate that the process in *Polychoerus* is probably not unlike that in most other forms; thus far, however, a sperm nucleus has not been seen at the stage when it bears a sperm aster. Gardiner (1898) figures such a stage (his Fig. 24) for *Polychoerus caudatus.* At the period of association of the maternal and paternal chromosome vesicles, two rod-shaped centrioles, with well developed asters, are frequently found (Fig. 6). Occasionally the two centrioles and asters are sufficiently far apart as to appear to be associated with separate groups of chromosome vesicles. These centrioles resemble those of the first cleavage metaphases in size and shape. The astral rays can be seen to originate along their entire lengths.

Curiously enough, I have thus far been unable to identify with certainty any centrioles (or asters) in the first or second meiotic divisions of the spermato-

cytes, which are found in other regions in the same serial sections of the same (hermaphroditic) animals that show such beautiful egg centrioles and asters. Fusiform spindles with tetrads or dyads are clearly seen, and the material appears to be excellent for a comparison of sperm chromosomes with egg chromosomes at corresponding meiotic stages. Large, distinct black dots (after haematoxylin staining) in the spermatocytes are undoubtedly the chromatoid bodies.

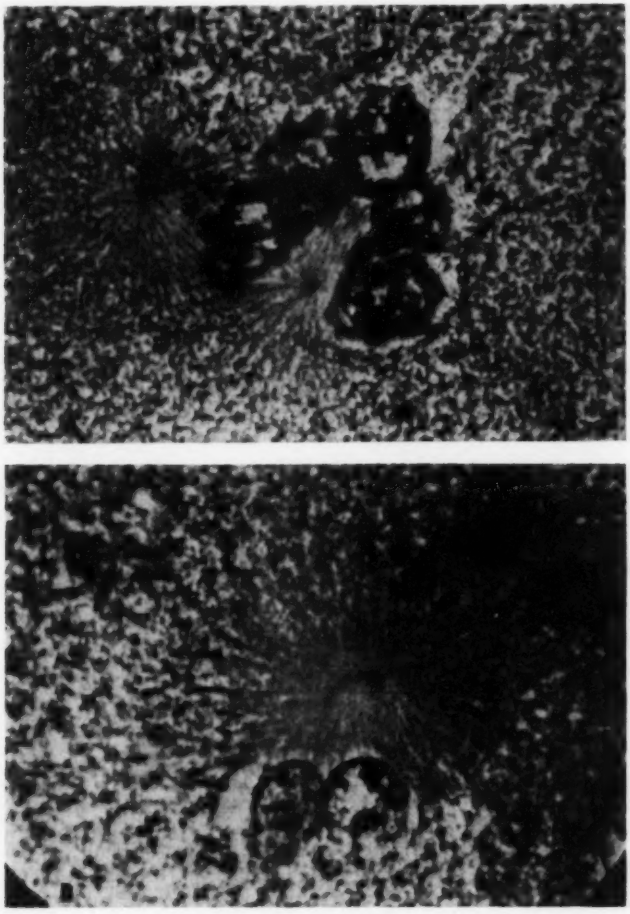


FIGURE 6, A. Beginning of cleavage diaster, and 6 of the 34 chromosome vesicles. The centriole between the vesicles is a rod, in plane of section. The other centriole is perpendicular to plane of section, and half is in the adjacent section. 910 ×.

FIGURE 6, B. Aster and one rod centriole of early cleavage diaster, associated with two chromosome vesicles, in another egg. Centriole foreshortened. Fixation: hot Heath's; stain: Heidenhain's iron haematoxylin. 910 ×.

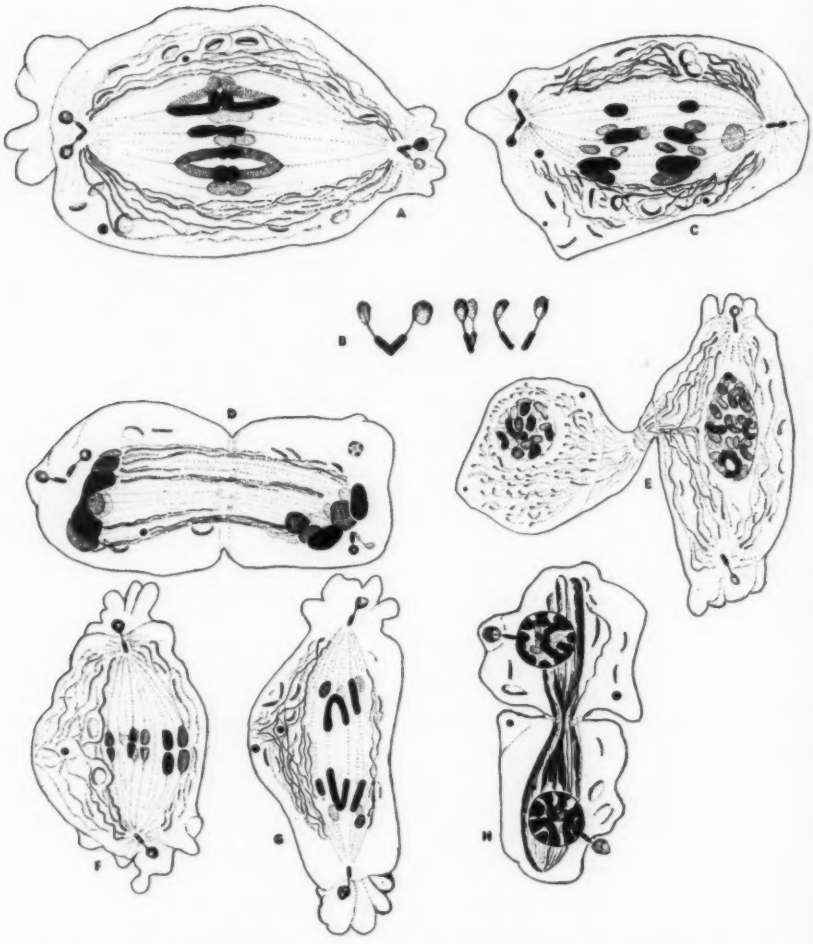
Discussion

Related observations made by others

The bivalent centrioles found in the first maturation division of *Polychoerus* are reminiscent of the V-shaped centrioles of the Gryllidae described in Johnson's excellent paper (1931), even though the V-shaped centrioles are oppositely directed as compared with those of *Polychoerus*. Johnson found that the centriole of the spermatogonium is a very short rod, scarcely longer than it is broad, frequently tilted at an angle to the long axis of the spindle. These centrioles are found at opposite ends of the nucleus *before* spindle formation. When the pachytene stage of the primary spermatocyte is reached, all the cells exhibit centrioles of a definite V-shape, directed apex inward, the tip of each limb in contact with the cell membrane. Johnson observed the migration and separation of these V-shaped centrioles in *living* material, and figured (his Figs. 83, A and B) the *rotation* of *one* of the two central bodies which had taken place over an interval of five minutes. He states (p. 124), "One central body nearly always lies in a plane at right angles to its fellow of the opposite pole, as seen in Fig. 35 [Figure 7,C of the present paper], in which the upper one is lying flat while the other is on edge. Rotation into this position is accomplished at various times during the prophase."

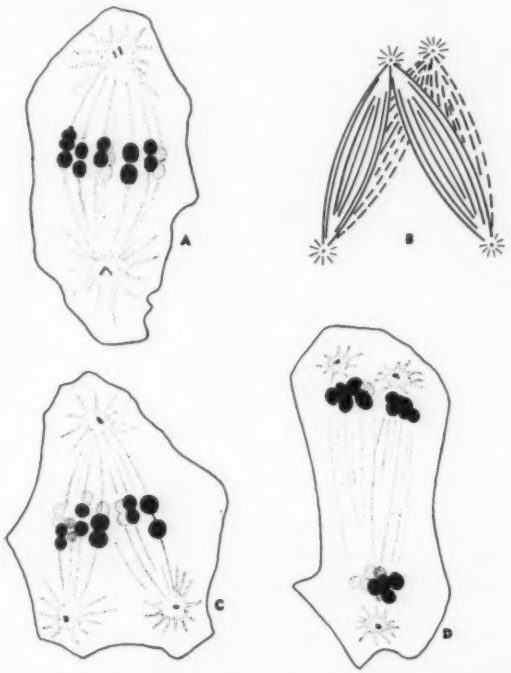
In *Oecanthus* and other Gryllidae studied, a split ocurs at the apex of the V at anaphase of the first meiotic division. This gives rise to centrioles of the second order which separate as short rods, each with its axial thread and terminal vesicle. These rods move apart 180° as the spindle for the next meiotic division is established. The second division spindle therefore forms at right angles to the first. However, Johnson (pp. 125–126) continues: "When the V-shaped central apparatus breaks into rods, the moieties move apart in that plane in which the arms previously diverged. Since the V's usually lie at right angles at the primary metaphase, the two secondary spindles will not only lie at right angles to the primary axis but also at right angles to each other. . . . That divisions of secondary cytes actually occur as I have indicated is quite evident in examination of second division cysts. If one cell is cut sagittally, its closest neighbor is usually cut transversely." (See Fig. 7,E in the present paper.) At metaphase of the second meiotic division the centriole rods of *Oecanthus* lie more or less directly in the longitudinal axis of the spindle, but sometimes, especially at anaphase, the elements are slightly tilted (Fig. 7,G in the present paper). Baumgartner (1929) described similar centriolar behavior during the spermatogenesis of another gryllid, *Nemobius fasciatus*.

Another equally interesting case is presented by Schrader (1941) in his paper on spermatogenesis in the earwig, *Anisolabis*. During the first meiotic division of the spermatocyte there are two centrioles visible at each spindle pole. He states (p. 136), "Each centriole is evidently a short rod. The axes of the two centrioles in each centrosome have no definite relation to each other and almost every possible variation has been encountered." Many of these cells divide normally (see Fig. 8,A of the present paper), while certain other cells exhibit a most interesting anomaly of spindle behavior (Figs. 8,C and D). As Schrader states (pp. 129–130), "In such cells the two centrioles at each pole, which normally remain closely associated in a single centrosome, begin to separate—usually just about the time that the metaphase is being established. . . . The precocious separation of the centrosomes occurs simultaneously at both poles of the cell involved. . . . The movement of



FIGURES 7, A through 7, H redrawn by Helen M. Costello from Johnson (1931), *Zeitschr. wiss. Zool.*, 140, with the permission of the publisher and author. All are of *Oecanthus nigricornis*. (A) First meiotic metaphase in primary spermatocyte. Centrioles at right angles to each other, with axial filaments and terminal vesicles. (B) Centrioles from first meiotic figures in front and side views. Daughter centrioles at right, after division of a V-shaped centriole. (C) First division, anaphase. The centriole at the right pole is in end view. (D) First division, telophase. Division and beginning migration of daughter centrioles. (E) Secondary spermatocytes at interphase. Adjacent daughter cells connected by spindle-bridge from the first meiotic division. Note the relation of the cell axes. The left cell is cut in transverse section. (F through H) Second meiotic division, metaphase, anaphase and telophase, respectively. Note the asymmetry of distribution of the chondrioconts in (F) and (G), and the telokinetic rotation of the nuclei and centrioles in (H). The dark dots are chromatoid bodies.

the centrosomes after separation is usually very definite. It is such that a plane passing through the original axis and the sister centrosomes at one pole is nearly always at right angles to one passing through the pair at the opposite pole. . . . The result is a quadripolar figure which, if it shows the centrosomes of one pole at the same focal level, will present the opposite pair of centrosomes optically superimposed on each other." (See Figs. 8,B and D of the present paper.)

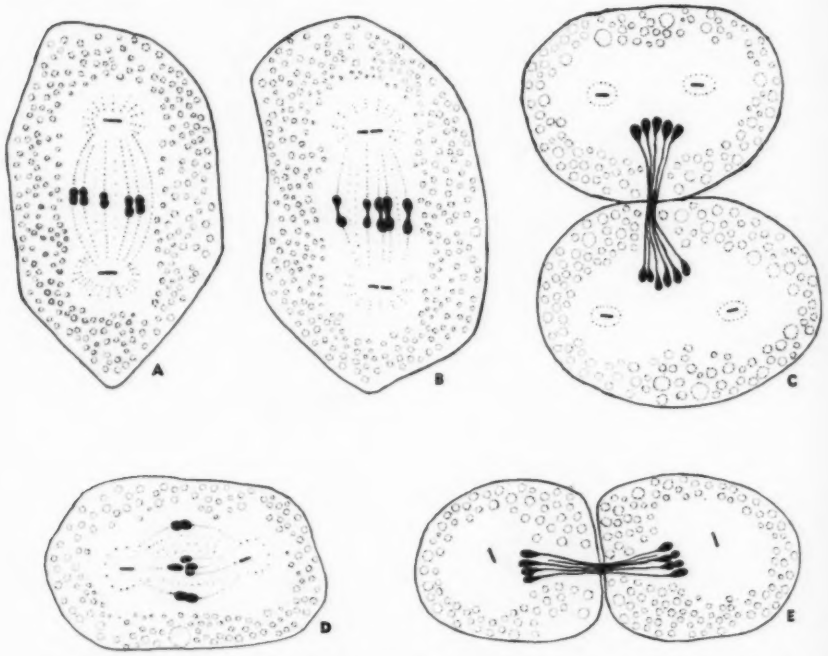


FIGURES 8, A through 8, D redrawn from Schrader (1941), *J. Morphol.*, **68**, with permission of the publishers and author. All are of *Anisolabis maritima*. (A) Normal first spermatocyte metaphase, in side view. (B) Diagram showing the four spindles that arise after precocious separation of the centrioles in two planes. (C) First meiotic metaphase with precociously divided centrosomes. Those at upper pole superimposed on each other and in a plane at right angles to those of lower pole. Only two of four spindles shown. (D) Telophase of first meiotic division, with precocious centrosomal division. The centrosomes at the lower pole superimposed on each other. Chromosomes in two groups at the lower pole.

The *Anisolabis* material of Schrader therefore shows a type of centriole behavior similar to that described by Johnson (1931) for *Oecanthus*, except that the *precocious separation* of the centrioles in the anomalous *Anisolabis* cells did not give rise (Schrader, p. 130) to the production of continuous fibers (*i.e.*, a spindle) between them.

In the spermatogenesis of the hemipteran *Gelastocoris*, as described by Payne

(1927), a different situation obtains. Payne states (p. 319), "[In the primary spermatocyte] the centriole is still single and a straight rod and lies at right angles to the long axis of the spindle [Figure 9,A in the present paper]. As the chromosomes constrict and begin to move apart the centriole divides transversely into two equal parts, which gradually separate as the chromosomes move toward the poles of the spindle [Figure 9,C in the present paper]. The second division follows



FIGURES 9, A through 9, E redrawn from Payne (1927), *J. Morphol.*, 43, with permission of publishers and author. All are of *Gelastocoris oculatus*. (A) Centriolar orientation at metaphase of the first spermatocyte division. (B) Early anaphase of the first spermatocyte division. The centrioles have divided. (C) Late anaphase of the first meiotic division. Centrioles have moved apart to become the centrioles for the second division. (D) Metaphase of second meiotic division. Centrioles have retained orientation in each daughter cell resulting from first division. (E) Telophase of the second meiotic division. Centrioles have turned and moved to one side in each daughter cell (spermatid).

without a nuclear reconstruction and the division figure is built around the two new centrioles which now lie more or less parallel to the long axis of the spindle [so that both spindles of daughter secondary spermatocytes lie in one flat plane]. As the chromosomes approach the poles in this division, the centriole moves to one side and lies here during the formation of the spermatid nucleus [Figure 9,E in the present paper]. . . ."

Thus we have two strikingly contrasting conditions of cell orientation in

Oecanthus and *Gelastocoris*, associated with differences in *centriolar orientation* and *behavior*.

Since in both these cases we are dealing with terminal divisions in spermatogenesis, the further significance is not immediately apparent.

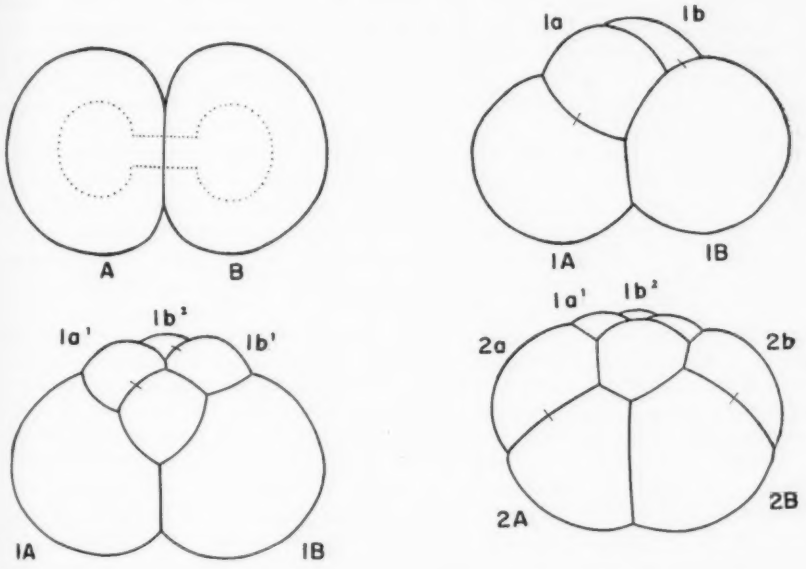


FIGURE 10. The early cleavages of *Polychoerus carmelensis* drawn from photomicrographs of living eggs. Egg membrane (chorion) and external jelly omitted. The cleavage of *Polychoerus carmelensis* differs in many details from that described by Bresslau (1909, 1933) for *Convoluta*, and by Gardiner (1895) for *Polychoerus caudatus*. (Upper left) Two-cell stage at the time of completion of the first cleavage furrow. The amphiaster is visible. (Upper right) Four-cell stage, side view. Two micromeres (1a, 1b) have been formed by a leiotropic second cleavage. (Lower left) Six-cell stage, side view, slightly tilted toward the observer. The members of the first duet of micromeres (1a, 1b) have divided by a dexiotropic cleavage, to give $1a^1$ and $1a^2$, and $1b^1$ and $1b^2$, and there has been some shifting of the blastomeres. (Lower right) Eight-cell stage, side view. The macromeres 1A and 1B have divided almost bilaterally but somewhat dexiotropically, producing 2a and 2b, and becoming 2A and 2B.

The possible significance of centriolar orientation

The *Polychoerus* material gives information not only on the orientation of centrioles during oögenesis, but also on their orientation for first cleavage. Omitting, for the moment, a discussion of the situation regarding the formation of the polar bodies, the possible significance of the relation of centriolar behavior to cleavage may be considered.

The first cleavage of the egg of *Polychoerus* is equal (or near-equal), giving rise to two cells, A and B (Fig. 10), with presumably identical cytoplasmic compo-

sition. The first cleavage plane bisects the large spindle and presumably passes through the animal pole, although this pole is not marked by any polar bodies. As I have pointed out briefly (Costello, 1960b), the polar bodies are not extruded but are intracellular, mark no axis, and eventually degenerate.

The right-angle orientation of the slightly curved centrioles to each other and to the spindle axis has obviously not influenced this first cleavage. The axes of the centrioles themselves, as also the single or duplicated state, bear no necessary relation to the spindle axis of that cleavage in which they are being observed. Evidence for this general conclusion is given, also, by the *Gelastocoris* centrioles, the axes of which are at right angles to the spindle axis in the first meiotic division and coincide with it for the second; by the *Oecanthus* centrioles, which are V's with apex toward the spindle at the first meiotic division and are rods roughly parallel to the spindle in the second; and by the *Polychoerus* centrioles which are slightly divergent paired rods (with apex away from the spindle) at the first maturation division and are longer curved rods at the second polar division.

However, it is immediately apparent what will happen at the second cleavage division of the *Polychoerus* egg if a similar pattern of inherent centriole behavior is followed. That is, the rod centrioles which are at right angles to each other in the first cleavage figure will each produce daughter centrioles and these will separate in a slightly oblique direction from each other, and at right angles in blastomere A as compared with blastomere B, generating the second cleavage spindle as they move apart. This oblique separation could be due to the slight curvature of the centriolar rods, especially if one daughter centriole of each blastomere were free to move more readily through the protoplasm toward (and to the left) of the animal pole, while the other might be unable to migrate as easily into the more densely yolk-packed vegetal region. It might also be related to the manner of origin (and hence orientation) of the daughter centrioles as they are produced. The members of the pairs of daughter centrioles in each blastomere will likewise be at right angles to each other, the upper one nearer the animal pole and at right angles to the lower one, near the middle of the vegetal hemisphere. The two oblique second cleavage spindles must likewise be assumed to be heteropolar, with the upper ends differing from the lower ends (like the heteropolar auxocyte of *Oecanthus* shown in Johnson's Fig. 5). This might be due to the type of protoplasm (animal versus vegetal) in which the ends lie. When this second cleavage is completed, we would then have, by a typical *leiotropic spiral cleavage*, a pair of micromeres, 1a and 1b, surmounting a pair of macromeres, 1A and 1B; this actually results at the second cleavage of the *Polychoerus* egg (Fig. 10, upper right).

If a similar pattern of centriole behavior is repeated at the third cleavage, with the formation of spindles determined by the axes of daughter centriole separations, the result would now be a *dexiotropic* cleavage for each cell that had arisen leiotropically. When this cleavage cycle is complete, we would have an 8-cell stage, with four micromere products ($1a^1$, $1a^2$, $1b^1$, $1b^2$), two second duet micromeres (2a, 2b) and two second generation macromeres (2A, 2B) (Fig. 10, lower right). We can thus explain the *regular alternation of leiotropic and dexiotropic* cleavages in the early development of this form, and, *presumably, in all other spirally cleaving forms*. (*Polychoerus* shows spiral cleavage by duets, practically all forms other than acoels show spiral cleavage by quartets. The mechanics of centriole behavior in the quartet type could be similar, but more complex.)

One can, therefore, set up the general hypothesis that the orientation of the centrioles with respect to each other and with respect to cell polarity, at any given division (meiotic, mitotic [or cleavage]) in which centrioles are normally present, determines the *original* position in which the daughter centrioles will separate from each other. This, in turn (provided there are not secondary re-orienting factors operating), *determines the main axis of the spindle for the next division, and hence* (barring the intervention of secondary factors), *the relative orientation of cells to each other* in the embryo, organ, or even organism. This orientation of the cells to each other should persist at least as long as the cells remain connected by the primary cell connective (spindle remnant or cell bridge), or until other forces move and re-orient them. The physico-chemical nature of the forces that orient the centrioles is as yet unknown (Schrader, 1947, 1953). It is clear that no simple explanation, in terms of electrostatic charges, nor of electromagnetic polarization, can account for rod centrioles orienting at right angles to each other.

A corollary of this hypothesis is the idea (already reasonably well established by others, as a working hypothesis) that the centrioles are the loci of origin of the orienting forces that spin out the astral rays and, at least, the continuous spindle fibers, and give rise to the distal centriole (blepharoplast) of the spermatid which spins out the axial filament of the sperm. There is likewise much evidence that the centrioles are the loci of forces orienting the mitochondria, etc. (Bowen, 1920, pp. 345-348; Johnson, 1931, p. 124).

Obviously, a theory dealing with centriolar behavior can have no direct application to cleavage patterns or cell orientations in forms where centrioles are lacking. (See also footnote 3.)

The apparent exceptions to the corollary view, in the forms where the spindle fibers originate from the kinetochores of the chromosomes (especially in anastral and acentriolar mitoses, and in forms showing diffuse chromosome fibers), may possibly be resolved by the suggestion (made years ago by Darlington, 1936; Schrader, 1936; Pollister, 1939; Pollister and Pollister, 1943; etc.) that the kinetochores contain centriolar material, or are, in effect, centrioles. This was especially beautifully demonstrated by Pollister's work on the supernumerary centrioles of certain gastropods. This matter could be resolved still more simply, if one assumes that the centrioles form the continuous spindle fibers, and the kinetochores, as slightly different counterparts of the centrioles, form the chromosomal fibers.

[3] It should be kept in mind that the role played by the centers or centrosomes in the mechanism of mitosis may vary in different organisms. Thus, Dietz (1959, *Zeitschr. Naturforsch.*, **14b**: 749–753) has shown that under experimental conditions in the insect, *Pales crocata*, a bipolar spindle may be formed even when one or both centrosomes are located in a distant part of the cell, and anaphase movements of the chromosomes may be quite normal. But how such an abnormal configuration of spindle elements affects cytokinesis is not reported by Dietz. Presumably, in *Pales*, also, irregularities in the division of the cell as a whole will result from such a displacement of the centers. Once the centrioles have done their work in spinning or orienting the fibers necessary for a given division, they may have no further function in that particular mitotic division. Hence, they could be removed and have the division continue. The kinetochores of the chromosomes might assume the entire function of fiber-formation under such circumstances, as they normally do in acentriolar mitosis. A more critical test of the effects of removal of centrioles would be to study the succeeding division (if this takes place) or to study the effect of centriole removal in a form where the centrioles clearly spin out the continuous fibers.

Further possible implications

This relation can be further checked by examination of the spindle axes in cleavage products of various other forms in which the centrioles are visibly rods of sufficient length so that their orientation can be ascertained.

If all the relations of centriolar patterns (it is obvious that there must be more than two) to cleavage patterns could be established, we could then use the more easily determined cleavage pattern as a means of ascertaining the centriolar pattern of the previous cell generation. This would enable us to predict what the centriolar pattern and behavior may be in forms in which the centrioles are too small (or too short, as cylinders) to ascertain this pattern directly. It may be possible, by merely glancing at a section of testis under the low power of the microscope, and finding that the axes of many secondary spermatocytes are at right angles to the axes of their nearest mates, to predict that the centrioles will be at right angles in the primary spermatocytes (as in *Oecanthus*). Where the spindle or cell axes lie parallel in adjacent secondary daughter cells, the *Gelastocoris* type of centriolar pattern must have obtained in the primary spermatocytes.

It is possible, of course, that there may be, in some forms, no constant relation between the daughter cells, but even in this case, the variability of arrangement may be attributable to the centriolar behavior. For example, in *Anasa tristis*, Paulmier (1899) found (p. 243) that ". . . the axes of the daughter spindles [in the two daughter secondary spermatocytes] were rarely parallel—the angles at which they lie being dependent upon the angles of the planes in which the two pairs of centrosomes [centrioles, in present terminology] move apart, these angles varying within 90°."

One further possible function of centrioles in determining movements of or within cells might be revealed through the investigation of the telokinetic movements in spermatids. In the spermatids (of the *Gelastocoris* type), when the centrioles move to one side of their respective nuclei (the same side) in both spermatids, as the interzonal connectives elongate, is there a correlated rotation of the two spermatid nuclei away from each other, one rotating clockwise, the other counterclockwise? Payne's paper does not show this stage.

The centriolar movements are presumably the first indications of the telokinetic movements, which have been described in more detail by Montgomery (1911), Bowen (1922) and Johnson (1931). Johnson (1931) states (p. 126), "Nuclei of sister spermatids may rotate into any position with respect to one another, about 55% turning at right angles to the direction of their sisters; i.e., if one is seen at the side of a spindle remnant, the sister nucleus is seen above or below the remnant. I do not believe that the direction of telokinetic movement in one cell has much effect upon the other product of the same division, but rather that the direction is controlled by the spatial relations of the cyst." But could it not be controlled by the positions of the centrioles in relation to the nuclei of the two daughter cells? Johnson's Figure 42 (Figure 7,H of the present paper) shows that the centriolar complex takes up exactly this position, so that the centriole-nuclear arrangement in one daughter spermatid is the opposite of that in the other. This is exactly opposite to the situation we should expect in *Gelastocoris*. We may tentatively suggest, therefore, that the centrioles supply the motive force that causes the interzonal fibers to continue to expand, to bring about the telokinetic rotation.

Early embryologists made many attempts to correlate the position of the first plane of cleavage or the median plane of the embryo with either the sperm entrance point or the copulation path of the migrating sperm nucleus. In various species these efforts met with some degree of success. The entrance point or sperm path may bear some constant relation, within these species, to the orientation of the sperm centriole, when it is finally in position to produce the centrioles of the sperm diaster, which become the centrioles of the first cleavage spindle. Hence, in this situation, also, the final orientation of the sperm centriole and the axis of separation of the daughter centrioles could be the significant feature in determining the first cleavage plane.

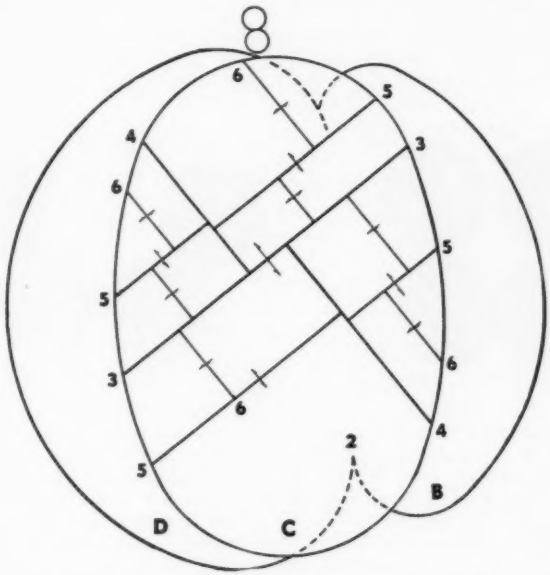


FIGURE 11. Diagram showing the positions of the successive cleavage planes from the third to the sixth (indicated by numbers), projected on the C quadrant of an unequally spirally cleaving egg (such as *Nereis*). Cleavages are alternately dexiotropic (odd-numbered) and leiotropic (even-numbered). Note that all dexiotropic cleavage planes are *at right angles* to all leiotropic planes. The A blastomere is omitted, and the second cleavage plane interrupted, for clarity. Displacements of the cells are not shown.

Patterns and transitions during spiral cleavage

The hypothesis that the orientation and behavior of centrioles may account for the pattern of spiral cleavage in forms like *Polychoerus,* where duets of micromeres are formed, can be readily extended to the quartet type of spiral cleavage, typical of *Crepidula, Nereis, Chaetopterus,* etc. In quartet type spiral cleavage, the spindle of any given cell lies approximately at right angles to those in adjacent cells in both adjoining quadrants at the 4- and 8-cell stages. The successive divisions of each cell, after the 4-cell stage, are alternately dexiotropic and leiotropic, during

the entire spiral period of cleavage. However, the first two cleavages in forms such as *Nereis* have the first two cleavage planes somewhat differently directed than the third through the sixth cleavage planes (see Figure 11). In the somewhat simpler situation of spiral cleavage by duets, as exemplified by the *Polychoerus* egg, it is only the first cleavage plane that is slightly anomalous, the others (until the transition to bilateral cleavage begins) being alternately leiotropic and dexiotropic.

There are thus two "critical" periods in spiral cleavage. The first is the period before micromere formation (examples: the first cleavage of *Polychoerus,* and the first two cleavages of *Nereis, Crepidula,* etc.). The second occurs when there is an inevitable transition from the strict alternation of dexiotropic and leiotropic cleavages to bilateral cleavages which herald the production of the bilaterally symmetrical acoel, polyclad, annelidan or molluscan larva. We know that in *Crepidula* (Conklin, 1897) the first cleavage is "prospectively" dexiotropic and the second is

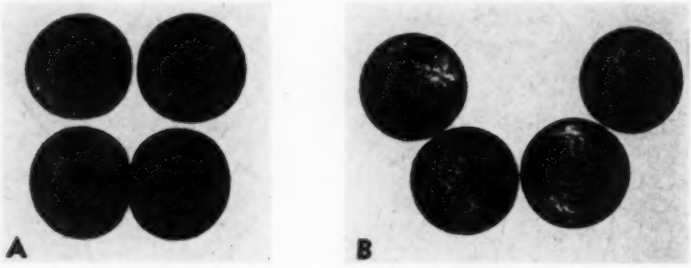


FIGURE 12. Four-cell stages of two living eggs of *Arbacia punctulata.* Magnification 340 ×. These eggs were fertilized in sea water containing Ishida (1936) hatching enzyme. They were transferred to Rulon calcium-low sea water 87 minutes later, and photographed 104 minutes after insemination. Note that the primary cell connective (spindle remnant) of the first cleavage connects the two lower blastomeres, and the two spindle remnants of the second cleavage connect the other daughter cells. All spindle axes are thus clearly indicated. (A) The initial position of the blastomeres. (B) Position after a very gentle tap on the coverglass.

"prospectively" leiotropic, as evidenced by the post-cleavage rotations of the protoplasmic areas and by the movements which lead to the formation of the cross-furrow. Perhaps someone will be able to pursue this subject, in a suitable material, and follow the behavior of the centrioles while the egg is undergoing the transition from spiral to bilateral cleavage. To my knowledge, there has been no other hypothesis suggested to attempt to account for the sequence of dexiotropic and leiotropic cleavages.

The primary cell connective

As cytokinesis is brought to completion and the two daughter cells are separated by the advancing cleavage furrow, there remains a spindle remnant of continuous and interzonal fibers, sometimes containing Zwischenkörper (Fig. 15). This spindle remnant, together with a thin layer of cortical cytoplasm and/or cell membrane, becomes the stalk or bridge connecting the daughter cells after division

is complete. In other words, the spindle remnant constitutes the major portion of the primary cell connective. This connective persists for a considerable period of time, if not permanently, in many materials. This is obvious to anyone who has ever isolated cleavage blastomeres free-hand with fine glass needles; the spindle remnant offers resistance to the needle and must be cut through. The axis of the spindle, of course, determined the point of attachment of the primary cell connective between the daughter cells.

If the eggs of *Arbacia punctulata* are treated to remove the membranes and examined at the 4-cell stage, as earlier demonstrated by Moore, (1930a, 1930b, 1945) for other echinoderm eggs, they show (Fig. 12) the primary cell connectives of the first cleavage spindle between the two middle cells, and the spindle remnants of the two second cleavage spindles at right angles to the first, between the other pairs of cells, exactly as pointed out by Moore (1930a, 1930b) for *Strongylocentrotus* and *Dendraster*. In the echinoderm, which is a radially cleaving form, these connectives are in the same plane, which is not the case in a spirally cleaving egg. The cell connectives are therefore a reflection of the pattern of orientation of the earlier spindles.

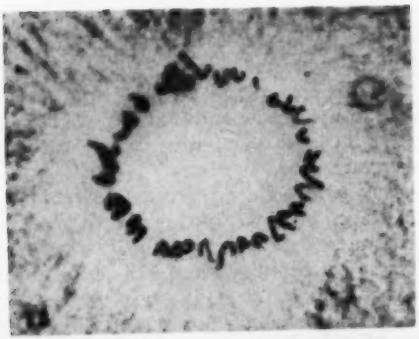
Kojima (1960) has also described the blastomere arrangements in the sea urchin egg, and has experimentally altered this relation by means of dinitrophenol. However, he does not interpret his results in terms of the centrioles, indicating only that the cause of the rearrangement is unknown. For really complex arrangements of multicellular bridges formed from spindle remnants among numerous cells during the oögenesis of *Vespa* (hornet), see the paper by Majiarski (1913).

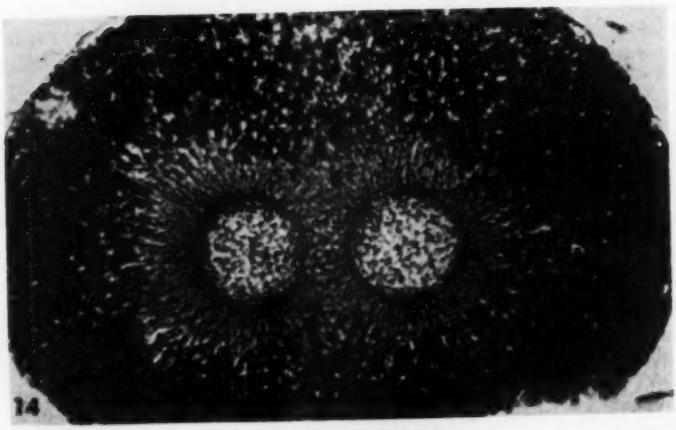
Sections through the eggs of *Polychoerus* just after the completion of the first division clearly show (Fig. 15) the nature of the primary cell connective. At the point where the furrow membrane meets the spindle remnant, Zwischenkörper may be distinctly seen. These have been described in many other cases, by early workers on cell division.

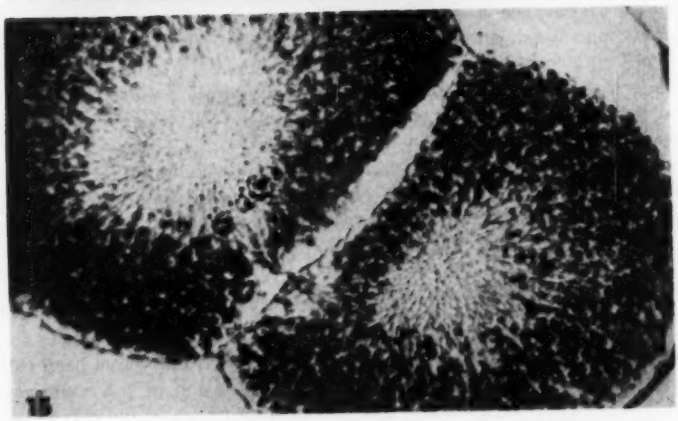
I believe that the primary cell connectives are far more important than is generally realized. In fact, the persistence of these connectives through a number of cell divisions may be a significant difference between Metazoa and colonial forms, on the one hand, and Protozoa on the other. In certain metazoan blastomeres the cell connectives are apparently totally absent. Fuliński (1916) described the complete separation of the blastomeres of the egg of *Dendrocoelum*, where the individual blastomeres lie dispersed in the external yolk. Here some other factor must have assumed the role taken by the primary cell connective in most forms. That the connectives are absent or can be relatively easily ruptured in some forms is indicated also by the experiments on the mechanical isolation of sponge cells (Wilson, 1907) and their subsequent reaggregation. Presumably, also, in many types of tissue cultures, the cells are free to wander.

Primary vs. secondary forces orienting spindles, and other problems

It is obvious that there are secondary forces which may re-orient a formed or forming spindle, and change the original relations which may have been established by centriolar orientation. The second maturation division of the ovum provides a striking demonstration of this fact. The second maturation spindle (of the secondary oöcyte), formed at the inner end of, and at right angles to, the axis of the first







FIGURES 13 through 15 are of *Polychoerus carmelensis.*

meiotic spindle (the position brought about by the separation of the inner daughter centrioles), must be rotated through 90° to become oriented perpendicular to the egg surface and give off the second polar body under the first. Similar forces may likewise be involved in orienting the first meiotic spindle perpendicular to the egg surface, at the animal pole. The nature of such polar forces is unknown, as is the nature of the forces that orient daughter centrioles with respect to each other. However, the maturation divisions of the ovum are terminal divisions—leading to no future role for the egg centrioles, in those cases in which the entering sperm centriole gives rise to the centrioles of the cleavage diaster. There may be other secondary re-orienting factors as well.

An equally important problem is whether there are differences in centriolar behavior patterns in cases where the daughter cells return to their resting interphase condition, as compared with rapidly dividing cells in which there is no nuclear reconstruction between successive cleavages. Conklin's extensive studies (1902) on centrosome and sphere during the cleavages of *Crepidula* may supply pertinent evidence here.

Division of cleavage centrioles

Because of the relative paucity of *Polychoerus* material available following the resting first cleavage metaphase stage, the stages of centriolar division have not yet been worked out. However, sufficient material was studied to suggest that the whole central apparatus of the *Polychoerus* egg may follow a pattern similar to that described by Vejdovský and Mrázek (1903) for the egg of the oligochaete, *Rhynchelmis*. In *Polychoerus* (Fig. 14), shortly after the eggs are laid, ". . . drastic changes occur at the central region of each aster, while the chromosomes are still at metaphase. The centriole disappears and the centrosomal region becomes a large sphere, which has acquired a definite boundary, not traversed by the rays, which remain outside it. Internally, the sphere is reticular, with numerous tiny granules, no one of which can be identified as a centriole. These spheres (centrospheres, astrospheres, or centrosomes), 40 μ or more in diameter, with their surrounding rays, are beautifully described as 'polar suns.'" (Costello, 1960a). In *Rhynchelmis*, there is a stage in the centrosomal cycle exactly corresponding to this "polar sun" stage. It is of transitory duration, and new daughter centrioles arise from within the reticulate mass where the old centriole had disappeared. The "disappearance" of a centriole may mean only that it has become unstable or unstainable, and has disappeared as a microscopically visible particle. Its reappearance may mean only that it has again become stainable, or of a size large enough to be microscopically detectable. This may complicate the study of the division cycle of the cleavage centrioles in *Polychoerus* by light microscopy, but it in no way negates the hypothesis outlined above.

FIGURE 13. Polar view of metaphase plate of resting first cleavage metaphase, showing all 34 chromosomes. Two were cut and one fragment of each was in the adjacent section. Fixation: Worcester's; stain, Heidenhain's iron haematoxylin. 990 ×.

FIGURE 14. Reticulate centrospheres and surrounding asters of 8-micron sagittal section of metaphase of egg just laid. Fixation: Flemming's; stain: Flemming's tricolor. 480 ×.

FIGURE 15. Zwischenkörper, spindle remnant, chromosome vesicles, etc., at late telophase of first cleavage, in 8-micron section. Fixation: Flemming's; stain: Flemming's tricolor. 480 ×.

Summary

1. The centrioles of the egg of *Polychoerus carmelensis,* at first meiotic metaphase, second meiotic metaphase, and resting first cleavage metaphase, are slightly curved rods which are usually oriented at right angles to each other and to the main axis of the spindle.

2. Centriole orientation and behavior in the spermatocyte divisions of Gryllidae and Hemiptera, as described by Johnson (1931) and Payne (1927), in relation to the arrangements of daughter cells, are compared with centriole orientation and predicted behavior in the egg of *Polychoerus.*

3. These considerations (on centriole orientation and behavior) constitute the basis for a new hypothesis, as follows:

a. The orientation of the centrioles at any given division determines the position in which the daughter centrioles will separate from each other.

b. The path of separation of daughter centrioles determines the position of the main axis of the spindle for the next division.

c. The axis of the spindle determines the relative positions of the daughter cells with respect to each other.

d. This arrangement of the daughter cells is maintained, for a time at least, by the primary cell connective, of which the spindle remnant is the significant portion.

e. These relations obtain in the absence of secondary intervening factors.

4. The inherent right-angle orientation of the centrioles at the two poles of the first cleavage spindle of *Polychoerus* is thus interpreted in causal relation to the alternating dexiotropic and leiotropic divisions in spiral cleavage.

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A CYTOCHEMICAL INVESTIGATION OF OÖGENESIS AND DEVELOPMENT TO THE SWIMMING LARVAL STAGE IN THE CHITON, CHITON TUBERCULATUM L.[1]

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The chitons have received very little attention from modern developmental biologists. Older accounts dealing with the morphology of chiton development have been published by Heath (1899), Hammersten and Runnström (1925), and Grave (1932). Yet for the chemical embryologist this group offers a number of advantages. Chitons may be readily induced to shed gametes in the laboratory, and artificially inseminated eggs develop well under laboratory conditions. Large numbers of eggs are produced by each individual, and although these eggs are surrounded by a rather ornate chorion, only a thin layer of jelly is present. Development in the species used in this study is quite rapid, requiring only about 12 hours at 26° C. to produce a swimming trochophore. In addition, like all molluscs, chitons are mosaic and exhibit the classical spiral pattern of cleavage.

The objective of this investigation has been to examine the pattern of distribution of nucleic acids, proteins and mucopolysaccharides during the development of the gonadal oöcyte, and during the subsequent development of the egg into the swimming larva. Since the mosaicism of the chiton egg must be dependent upon its physico-chemical organization, by examining oögensis cytochemically one might hope to identify those aspects of the organization of the egg which are causally related to the mosaic nature of its development.

Further, a consideration of the distribution of the macromolecular substances during development could yield information about the patterns of synthetic activity in molluscan mosaic development as compared to mosaic development in other phyla and also to regulative development.

Materials and Methods

The chitons used in these studies were collected in the vicinity of the Bermuda Biological Station, St. George's West, Bermuda, and identified as *Chiton tuberculatum* L. Specimens were removed from intertidal rocks at low tide and maintained in moist but not filled sea water tanks until the second evening after their collection. Individual animals were then placed in fingerbowls and covered with

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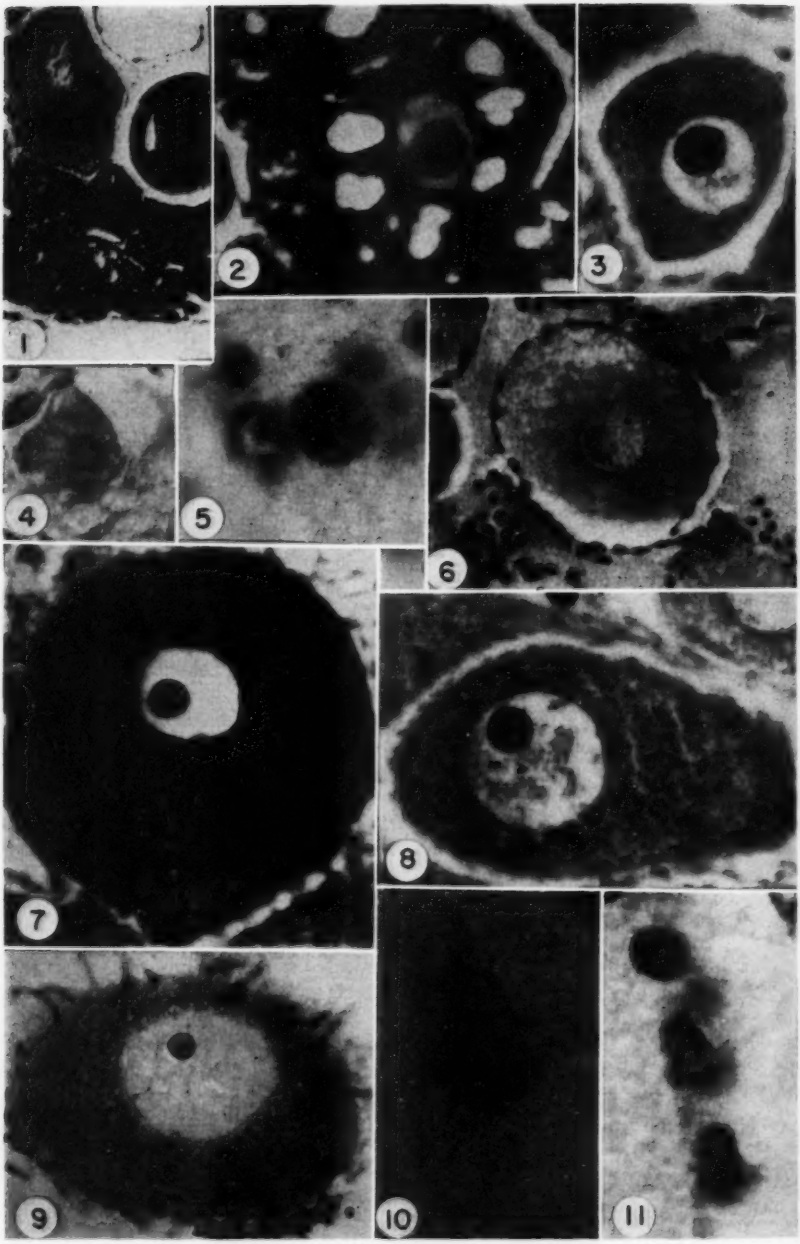


FIGS. 1–11.

sea water. Ovulation or the release of sperm ensued two to three hours later. Eggs were then inseminated with sperm suspension and allowed to develop. Development was observed with a binocular dissecting microscope, and embryos were removed for fixation at the desired stages.

For studies of oögenesis, the ovaries were removed from fresh specimens and fixed in appropriate fixatives. Acetic acid-ethanol (1:3) fixation was used for material to be stained for nucleic acids, Lillie's (1954) calcium acetate formalin for material to be stained for mucopolysaccharides and proteins, and Bouin's for material to be stained in mercuric bromphenol blue for proteins.

The pH 4.0 azure B bromide method of Flax and Himes (1952) was used for demonstrating RNA in sections which had been pre-treated with DNAase; Einarson's (1949) gallocyanin-chromalum method was used for staining DNA in sections which were pretreated with RNAase. Crawley *et al.*'s (1956) alcian blue-periodic acid Schiff (AB-PAS) method, which distinguishes between acid mucopolysaccharides and other polysaccharides or substances containing 1,2-glycol groups, was used for visualizing mucopolysaccharides. Some sections of embryos were stained with either alcian blue or with the PAS reaction alone. Some control sections were incubated in 0.1% α-amylase prior to staining with the PAS reaction to remove glycogen. Mazia, Brewer and Alfert's (1953) mercuric bromphenol blue (MBB) method for reactive groups of proteins was employed on Bouin-fixed sections. The picro-naphthol blue black-brilliant purpurin-azofuchsin (NBB-BP-Az) connective tissue method of Lillie (1954) was used without the conventional nuclear staining on calcium acetate formalin-fixed material in an attempt to distinguish between various protein components of the cells. Each of the three acid dyes used in this mixture stains only protein structures, probably by binding to the sites of dissociable basic groups. Some calcium acetate formalin-fixed sections were stained for protein tryptophan by Adam's (1957) dimethylaminobenzaldehyde method.

The eggs and embryos were partially dehydrated in 70% and 95% ethanol and then dehydrated in tertiary butanol. They were infiltrated with first a slush of

FIGURE 1. Chiton ovary, low power photomicrograph. Azure B bromide after DNAase. 300 ×.

FIGURE 2. Early stage one oöcyte with large but not heavily basophilic nucleolus. Azure B bromide after DNAase. 1920 ×.

FIGURE 3. Stage one oöcyte. Mercuric bromphenol blue. 1920 ×.

FIGURE 4. Early stage one oöcyte showing almost complete absence of cytoplasmic staining. Protein tryptophan method. 750 ×.

FIGURE 5. Smallest recognizable oöcyte stained for DNA; note nucleolus-associated heterochromatin and coarse nuclear chromatin. Gallocyanin-chromalum after RNAase. 4800 ×.

FIGURE 6. Larger stage one oöcyte surrounded by accessory cells. Gallocyanin-chromalum after RNAase. 1200 ×.

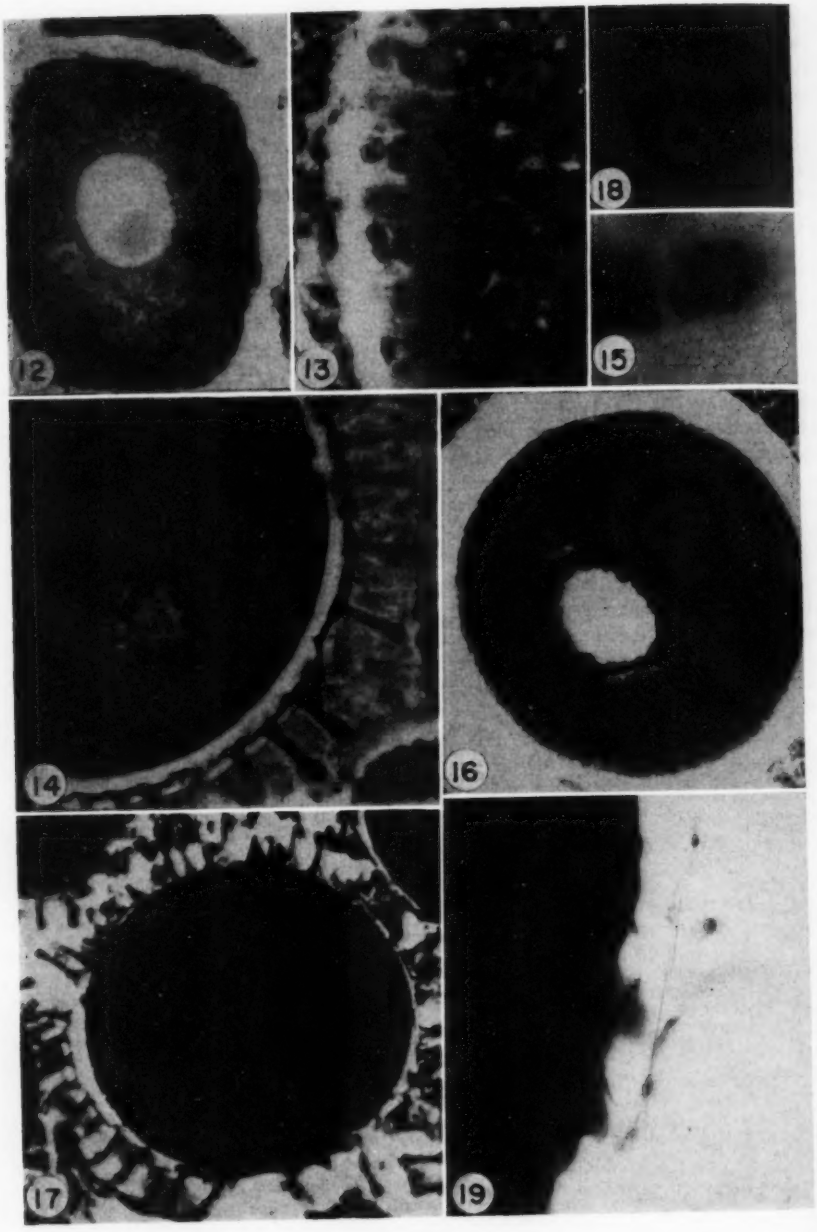
FIGURE 7. Stage two oöcyte with somewhat diminished cytoplasmic basophilia and relatively smaller nucleolus than stage one oöcytes. Azure B bromide after DNAase. 1200 ×.

FIGURE 8. Stage two oöcyte showing distribution of proteins. Mercuric bromphenol blue. 1200 ×.

FIGURE 9. Terminal oöcyte showing nucleolus and protein yolk granules stained by protein tryptophan method. 768 ×.

FIGURE 10. Stage two oöcyte nucleus in pachytene. Gallocyanin-chromalum after RNAase. 1200 ×.

FIGURE 11. Nuclei of oöcyte accessory cells. Gallocyanin-chromalum after RNAase. 4800 ×.



FIGS. 12–19.

equal parts of tertiary butanol and Tissuemat, and finally in pure Tissuemat. This treatment is preferable to conventional infiltration with xylol or toluol for yolky material. Eggs, embryos and ovaries were embedded in 56–58° C. m.p. Tissuemat and serially sectioned at 5 μ except for the specimens to be stained for DNA, which were sectioned at 15 μ.

Observations

Oögensis. A germinal epithelium surrounds the chiton ovary. The smallest oöcytes were found on the periphery of the ovary; the oöcytes increase in size toward the inner portion of the ovary (Fig. 1). Three different stages could be recognized in the development of the chiton oöcytes. In the first stage the oöcyte underwent its greatest relative increase in size, and this was also the stage in which the principal elaboration of cytoplasmic RNA occurred; in the second stage yolk synthesis was initiated and a chorion was formed. Except for the nuclei which were in diplotene and diakinesis of meiosis, third stage oöcytes were indistinguishable from freshly ovulated eggs.

The cytoplasm of stage one oöcytes was intensely basophilic. The nuclei contained a single basophilic nucleolus which occupied a large proportion of the nuclear volume (Fig. 2). In sections stained with mercuric bromphenol blue for basic groups of proteins, the relative intensity and distribution of staining were similar to that of basophilia due to RNA (Fig. 3). Differential staining of various protein structures was obtained with the (NBB-BP-Az) method: nucleoli were stained red by azofuchsin, nuclear proteins brown by brilliant purpurin, and cytoplasmic proteins red by azofuchsin. Toward the end of the first stage, however, there was a change in the staining behavior of the oöcyte cytoplasmic proteins. Instead of binding the red dye, azofuchsin, they bound the brown dye, brilliant purpurin. Again the distribution of staining was similar to that of RNA basophilia. The protein tryptophan method produced only weak staining in the cytoplasm of stage one oöcytes (Fig. 4). The cytoplasm of these oöcytes also contained very little glycogen; the cytoplasm was faintly PAS-positive, and this could be abolished by prior digestion in α-amylase. The oöcyte nucleoli were slightly PAS-positive, and nucleolar staining persisted with this method through all three stages until the nucleolus disappeared. No alcian blue staining was observed in stage one oöcytes which could not be prevented by prior incubation in RNAase.

Figure 12. Chiton oöcyte showing distribution of alcian blue-stainable material (dark) and PAS-positive material (light) at stage of chorion formation. Alcian blue-PAS. 1920 ×.

Figure 13. Evagination of chorionic processes from chorion, pushing accessory cells away. Mercuric bromphenol blue. 3000 ×.

Figure 14. Chorionic processes fully developed with "stalk" and terminal "bud." Picronaphthol blue black-brilliant purpurin-azofuchsin. 1200 ×.

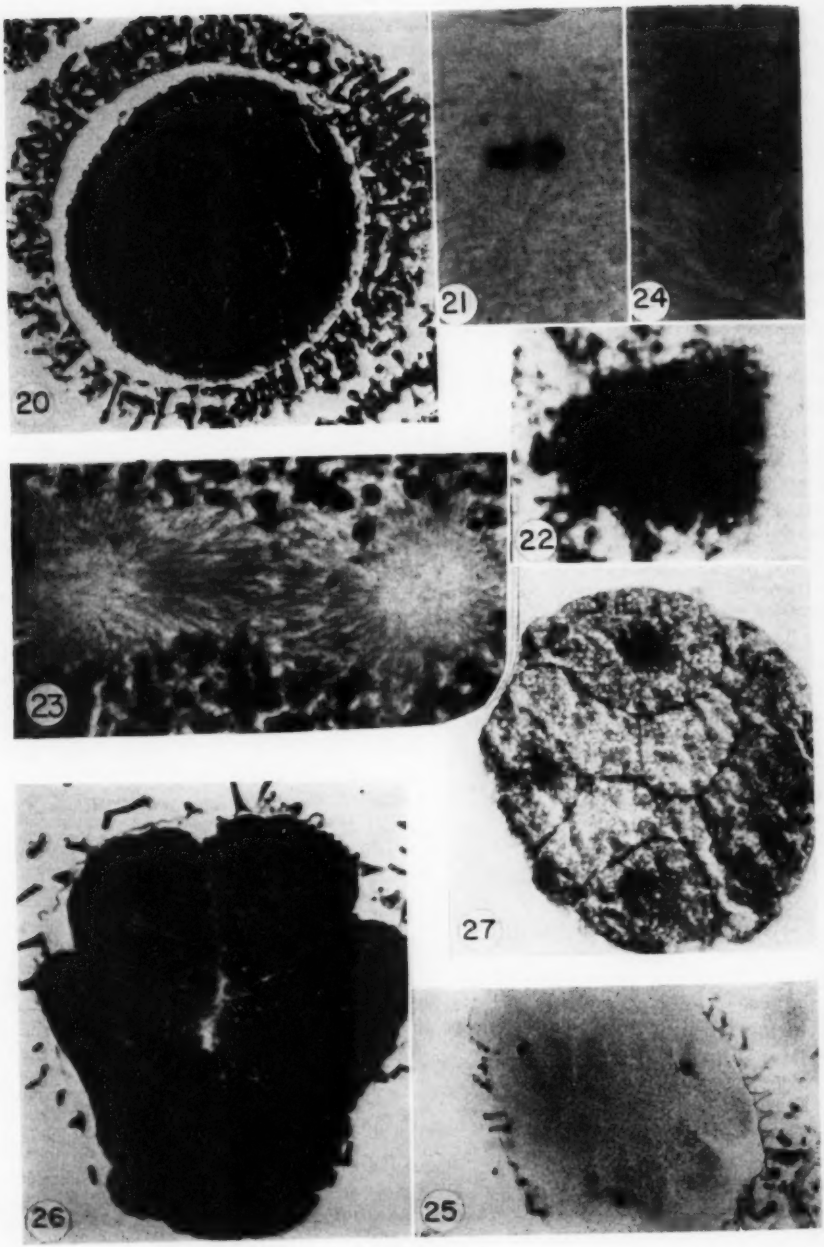
Figure 15. Top view of "bud," showing six radially symmetrical points and a center point. Picronaphthol blue black-brilliant purpurin-azofuchsin. 4800 ×.

Figure 16. Stage three oöcyte, showing reduced cytoplasmic basophilia except around nuclear membrane and absence of nucleolus. Azure B bromide after DNAase. 1600 ×.

Figure 17. Stage three oöcyte; cytoplasm is filled with uniform protein yolk granules. Mercuric bromphenol blue. 600 ×.

Figure 18. Stage three oöcyte nucleus stained for DNA, showing postpachytene chromosomes adherent to nuclear membrane. Gallocyanin-chromalum after RNAase. 1920 ×.

Figure 19. Cortical layer of stage three oöcyte is stained by alcian blue, the endoplasm beneath is filled with PAS-positive protein yolk granules. Alcian blue-PAS. 4800 ×.



FIGS. 20–27.

The chromosomes were somewhat condensed in very small stage one oöcytes. The nucleolus was surrounded by nucleolus-associated heterochromatin which, like the chromosomes, was stained by the gallocyanin-chromalum method for DNA (Fig. 5). In the later phases of stage one, the oöcytes became surrounded by a layer of somewhat flattened oöcyte accessory cells (Fig. 6).

In the second stage of oöcyte growth the concentration of cytoplasmic basophilia was reduced and the nucleolus occupied a relatively smaller proportion of the nucleus (Fig. 7). In addition to the RNA-associated proteins which were present in stage one, small granules of protein yolk appeared which were deeply stained by mercuric bromphenol blue (Fig. 8). With the (NBB-BP-Az) method, the yolk granules were stained by azofuchsin while the RNA-associated proteins were stained by brilliant purpurin; both the nucleolus and nucleoplasm continued to stain as in stage one. The yolk granules were strongly stained by the protein tryptophan method (Fig. 9), and were also strongly PAS-positive. Again, within the oöcyte there was no alcian blue staining which was not removable by prior digestion in RNAase. The stage two oöcyte chromosomes were in pachytene, and were adherent to the nuclear membrane (Fig. 10).

The second stage oöcytes were chiefly concerned with yolk synthesis, but outside the oöcyte, the oöcyte accessory cells were involved in the construction of a chorion. While these cells were somewhat flattened in stage one, they proliferated and eventually formed a single layer of rounded cells around the stage two oöcyte (Fig. 11). A chorionic membrane was then laid down around the oöcyte beneath the oöcyte accessory cells. The chorion itself was PAS-positive (Fig. 12) and was lightly stained by both mercuric bromphenol blue and by azofuchsin in the (NBB-BP-Az) method. Then from beneath the oöcyte accessory cells, hollow hydranth-like processes evaginated from the chorion (Fig. 13). These processes grew outward from beneath the oöcyte accessory cells. When fully developed, the processes consisted of a distal hydranth-like "bud," a stalk, and a basal region which opened into the space between the chorion and the oöcyte (Fig. 14). The "bud" consisted of six radially symmetrical points and one center point (Fig. 15). When the development of the processes was completed, the accessory cells were no longer in evidence.

The cytoplasm of stage three oöcytes was considerably less basophilic than the cytoplasm of stage two oöcytes. The basophilia was distributed between the large protein yolk granules except in the region of the nuclear membrane where it seemed to be accumulated in a concentric pattern (Fig. 16). The nucleoli had disappeared

FIGURE 20. Ovulated egg. Picronaphthol blue black-brilliant purpurin-azofuchsin. 768 ×.

FIGURE 21. Polar body division stained for DNA. Gallocyanin-chromalum. 4800 ×.

FIGURE 22. Polar body division, showing staining of the meiotic apparatus and fine cytoplasmic granules surrounding the apparatus. Mercuric bromphenol blue. 4800 ×.

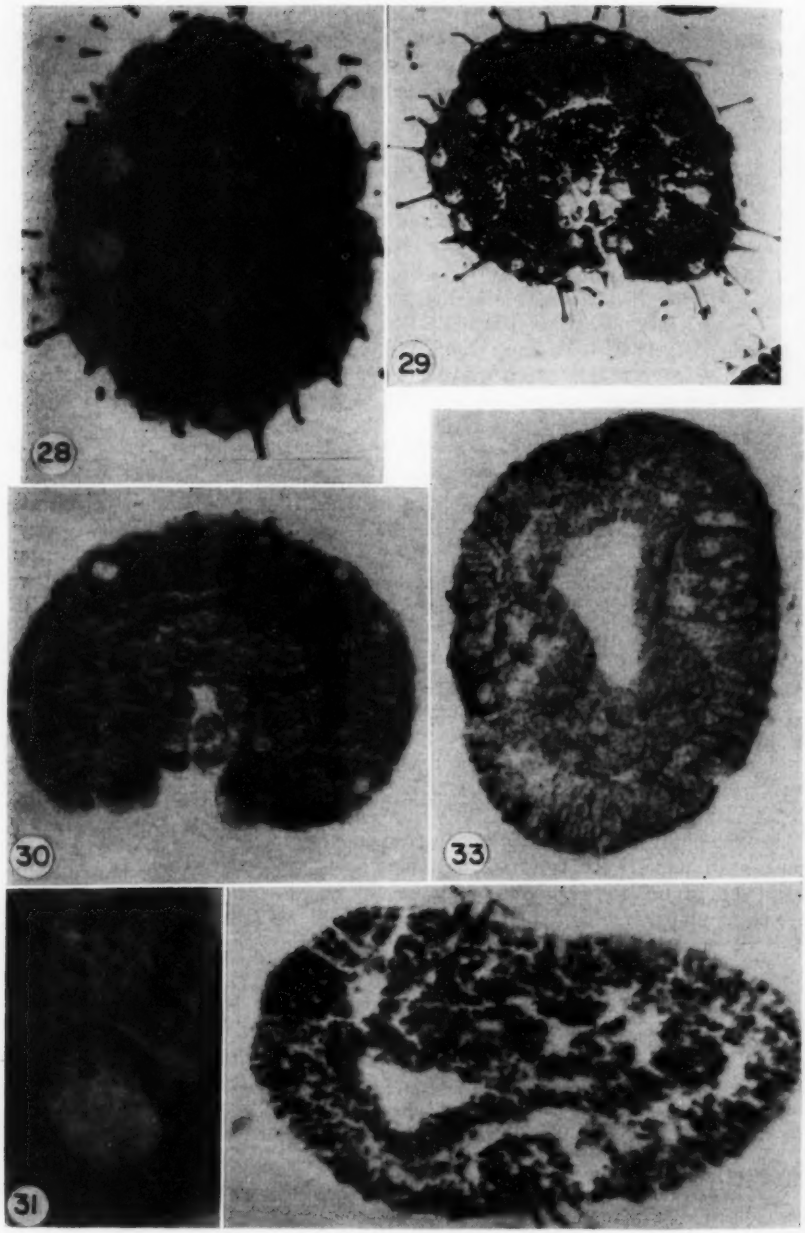
FIGURE 23. Mitotic apparatus of an early cleavage figure stained for protein. Mercuric bromphenol blue. 3840 ×.

FIGURE 24. Cleavage figure stained for DNA; note spindle is longer and narrower than polar body spindle. Gallocyanin-chromalum after RNAase. 4800 ×.

FIGURE 25. Spiral cleavage pattern. Gallocyanin-chromalum after RNAase. 800 ×.

FIGURE 26. Early cleavage embryo stained for protein; note equal density of protein yolk granules in all blastomeres. Mercuric bromphenol blue. 768 ×.

FIGURE 27. Intermediate cleavage stained for RNA; note equal distribution of cytoplasmic basophilia; dark nuclear zones are mitotic apparati. Azure B bromide after DNAase. 768 ×.



FIGS. 28–33.

in most stage three oöcyte nuclei. The cytoplasm was filled with protein yolk granules of uniform size which gave staining reactions identical to those observed in the smaller growing granules of stage two (Fig. 17). In the nucleus, the nucleolus had disappeared, and the post-pachytene chromosomes were observed adherent to the nuclear membrane (Fig. 18). Beneath the chorion, a granular cortical zone appeared which was stained by alcian blue (Fig. 19), presumably acid mucopolysaccharide material.

The chorionic processes had completed their outward growth by the beginning of stage three, and the oöcyte was coated with mucoprotein material. This material surrounded the oöcyte from the chorion to just beyond the distal "buds" of the chorionic processes (Fig. 20). This substance was lightly stained by both mercuric bromphenol blue and azofuchsin in the (NBB-BP-Az) method, faintly PAS-positive, and contained some granules which were deeply stained by alcian blue.

Early development. After ovulation, the first meiotic division occurred (Fig. 21). Shortly after fertilization, a second division followed, but it was not possible to determine the sequential relationship between the second polar body division, and the entrance of the sperm. The cytoplasmic distribution of both protein yolk granules and RNA-basophilia was identical with that observed in stage three oöcytes except in the region of the meiotic apparatus. Here yolk granules were pushed aside and the spindle was surrounded by smaller granular protein material (Fig. 22). Basophilia was associated with the spindle.

The mitotic apparati of cleaving embryos were stained by the mercuric bromphenol blue method for proteins (Fig. 23). Cleavage occurred by normal mitosis (Fig. 24), and exhibited the spiral pattern (Fig. 25). Both protein yolk and basophilia were evenly distributed within the cells (Figs. 26 and 27). Since the macromeres were larger they received more material, but not through any obvious differential segregation of material. No nucleoli were formed in the cleavage blastomere nuclei. However, there was considerable difference in the size of macromere and micromere nuclei (Fig. 28).

By the time of gastrulation the density of protein yolk granules was greater in the endodermal cells which had been derived from the invaginated macromeres (Fig. 29). No local increases in basophilia were observed (Fig. 30), but very small nucleoli appeared in the ectodermal cells which later became the prototroch or apical tuft. These nucleoli were only faintly basophilic in the gastrula (Fig. 31). The slight difference in degree of basophilia between the two primary layers is probably due to differences in cell size.

FIGURE 28. Intermediate cleavage stained for proteins; note equality of distribution of protein yolk granules and disparity in size of nuclei of the various blastomeres. Mercuric bromphenol blue. 768 ×.

FIGURE 29. Gastrula stained for protein; somewhat heavier concentrations of protein yolk granules in the endoderm. Mercuric bromphenol blue. 600 ×.

FIGURE 30. Gastrula stained for RNA; with the exception of division figures, basophilia not significantly different in the primary layer. Azure B bromide after DNAase. 768 ×.

FIGURE 31. Prototroch cell showing increased cytoplasmic basophilia and a small nucleolus. Azure B bromide after DNAase. 4800 ×.

FIGURE 32. Trochophore larva showing distribution of protein; note yolk in the larval gut. Picronaphthol blue black-brilliant purpurin-azofuchsin. 768 ×.

FIGURE 33. Trochophore larva showing distribution of RNA; the prototroch and apical tuft cells are basophilic. Azure B bromide after DNAase. 768 ×.

In the swimming larva, protein yolk was most dense in the endodermal cells of the trochophore gut (Fig. 32). The cytoplasm of the cells of the prototroch and apical tuft was intensely basophilic, and their nucleoli were somewhat more heavily stained than in the gastrula (Fig. 33). In the other cells of the trochophore, there was no local increase in basophilia, and no nucleoli were present.

Discussion

The patterns of appearance and distribution of RNA in chiton oöcytes were similar to those described in other molluscan species (see Raven, 1958). The greatest relative volume increment occurred in the first stage of oöcyte growth. This was also apparently the stage in which the principal synthesis of cytoplasmic RNA occurred. In the successive stages of oöcyte growth cytoplasmic basophilia decreased, suggesting that either RNA synthesis had ceased entirely or that the rate of its synthesis was not commensurate with the oöcytes' growth in volume. The oöcyte nucleoli had also reached their maximum size and intensity of basophilia by the end of the first stage. Ranzoli (1953) reported a similar situation in the oöcytes of the gastropod *Patella*. RNA synthesis and nucleolar growth apparently reached a peak just prior to the initiation of protein yolk synthesis. In all of the published accounts of RNA distribution in relation to oöcyte growth in molluscs, a progressive decrease in cytoplasmic concentration has been noted as the oöcytes increased in size. RNA synthesis appears to be chiefly restricted to the first stage of oöcyte growth prior to protein yolk synthesis in *Chiton tuberculatum*, and if some cytoplasmic RNA synthesis continues after the nucleoli start to become depleted, as suggested by Swift *et al.* (1956), it is not sufficient to maintain cytoplasmic RNA concentrations at the level observed in the first stage of oöcyte growth during the subsequent stages. Further, the results of this investigation suggest that very little RNA synthesis occurs during the development of the chiton to the trochophore stage. The little synthesis that did occur was limited to a single kind of cell: namely, the cilia-bearing cells of the prototroch and apical tuft.

The single incidence of such a pattern of RNA distribution during oögenesis and early embryology would hardly represent sufficient grounds for proposing a general mechanism in mosaic development. Recently, however, Cowden and Markert (unpublished data) have investigated the distribution of RNA during the development of ascidian, *Ascidia nigra*, and here also no evidence of new synthesis of RNA was found at any stage from the second stage of gonadal oöcyte growth to the initiation of metamorphosis in the swimming larva. This is not true of all molluscan or ascidian embryos. Minganti (1950) reported that basophilia increased during the development of the gastropod *Lymnaea*, and according to Raven (1958), nucleoli initially appear in the nuclei of 24-cell stage embryos of *Lymnaea*. More recently Mancuso (1959) has described the appearance of nucleoli and increasing cytoplasmic basophilia in cells taking part in major morphogenetic events such as neural tube formation or myogenesis in the ascidian, *Ciona*. In mosaic development, however, primary differentiation does not occur at or just prior to gastrulation, but is fixed at the latest by the four-cell stage. While it is probable that the new synthesis of RNA may be initiated at any time during the later course of development, starting earlier in some species than in others, the production of new RNA does not appear to play any role in primary differentiation of molluscan or ascidian

embryos. To do so, synthesis would have to be initiated before the second cleavage, and none of the usual manifestations of RNA synthesis have been detected in early cleavage stages in any of the molluscan or ascidian embryos for which adequate cytochemical studies are available. If RNA has any function in primary differentiation, it must depend on RNA produced during oöcyte growth. Such a function could be possible if there were a heterogeneity in the distribution of qualitatively different kinds of RNA in the mature ovum. In this way the different kinds of RNA could be segregated into the blastomeres at cleavage. Many of the classical experiments on the effects of centrifugation on the development of mosaic eggs suggest that some form of cytoplasmic "structuring" is present in these eggs.

Protein yolk synthesis occurred primarily in the second stage of chiton oöcyte growth. These protein yolk granules were considerably richer in protein tryptophan than the other cytoplasmic proteins. Thus the synthetic activities of the oöcyte fell into two distinct phases: the elaboration of RNA-proteins and the production of specialized cytoplasmic proteins. Since the oöcyte is a very specialized cell, this dichotomy may not necessarily represent the patterns of synthetic activity of cells in general, but is probably true of other cell types which produce a particular kind of protein in quantity, *e.g.*, the mammalian reticulocyte. It is also interesting to note that the staining affinity for acid dyes of the cytoplasmic RNA-associated proteins was altered as the oöcytes approached the period of protein yolk synthesis. Since these dyes in acid solutions are generally bound at the sites of dissociated basic groups of proteins, such a shift in affinity would not be expected to depend on differences in kinds of reactive groups but rather on differences in the arrangement of dissociated reactive groups. According to Baker (1958), differential binding of acid dyes in mixtures probably depends on the size of the dye molecule, the spacing of its reactive groups, the degree to which the dye molecules tend to form aggregates, and the structural arrangement of the proteins in question. A shift in affinity, such as that observed in the later stage one chiton oöcytes, could indicate an alteration in the structure of the RNA-protein complex. Both Himes (1958) and Love and Liles (1959) have developed methods for detecting differences in the RNA-protein complex by alterations in the capacity of the RNA-moiety to bind basic dyes under certain conditions. Such alterations can also apparently be detected by staining methods designed to attach to the protein moiety. By the end of the first stage, the generation of cytoplasmic ribonucleoprotein (RNP) had essentially been completed, and an alteration in the RNP complex in preparation for protein synthesis might occur. This is particularly interesting in the light of recent electron microscope studies which have indicated that Golgi zones might be specializations of the endoplasmic reticulum. Bolognari (1960) has convincingly demonstrated that protein yolk synthesis in the oöcyte of two molluscan species occurs within the Golgi zones, and that these Golgi zones are composed of concentric lamallae which contain dark granules of about 300 Å diameter.

The functions of the oöcyte accessory cells and chorionic processes deserve some comment because of their unusual nature. The processes themselves seem to be hollow modifications of the chorion which presumably increase its surface area and facilitate exchange. The oöcyte accessory cells were involved in the construction of the process since each process grew outward into an accessory cell and the accessory cells were absent when the processes were completely formed. The oöcyte

accessory cells were also probably involved in the construction of the chorion. When the accessory cells achieved their maximum size and number, the chorion appeared between the accessory cells and the cortex of the oöcyte. The role of these cells may not necessarily involve the production of chorionic material since there was an abundance of cortical PAS-positive and protein material in the oöcyte, but they may produce a tanning agent which hardens the chorionic material. In other invertebrates which have tough chorions such a mechanism is involved. Monné (1955) and Smyth (1956) have investigated chorion formation in a number of invertebrates, and some form of quinone tanning was involved in each case.

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Summary

1. Oöcyte growth and development and the subsequent development of the egg to the swimming larva stage were investigated in the chiton, *Chiton tuberculatum*, using topological cytochemical methods for nucleic acids, proteins and mucopolysaccharides.

2. The growing oöcytes exhibited two phases of synthetic activity: the first phase was chiefly concerned with RNA production and the second phase with protein yolk synthesis.

3. A probable alteration in the cytoplasmic RNA-protein complex prior to the initiation of yolk synthesis was detected by an alteration of the affinity of RNA-associated proteins for binding acid dyes in mixtures.

4. Oöcyte accessory cells were probably responsible in some way for chorion formation. After the chorion was formed, ornate hollow processes grew out of the chorion, each beneath an accessory cell. When development of the processes was completed, the accessory cells had disappeared.

5. There was no evidence of new synthesis of RNA in any of the cells of the developing embryo except the cells of the prototroch and apical tuft. This applies to the complete developmental history of the oöcyte from the cessation of RNA synthesis prior to protein yolk formation in the oöcyte to the swimming trochophore larva.

6. Since primary differentiation occurs in mosaic embryos in the absence of synthesis of new RNA, the possibility that primary differentiation is controlled by the RNA produced in the oöcyte was discussed.

7. Although there was no differential segregation of protein yolk into the blastomeres—both RNA-basophilia and protein yolk being evenly distributed in all blastomeres—the posterior blastomeres received a larger proportion of yolk material than the micromeres. This was particularly evident in the yolk-laden cells of the trochophore gut.

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PHYSIOLOGY OF THE VINEGAR EEL, TURBATRIX ACETI (NEMATODA). I. OBSERVATIONS ON RESPIRATORY METABOLISM[1]

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The demonstration of the Krebs tricarboxylic acid cycle in many vertebrate tissues, and failure to confirm reports of its absence in others, have led to the conclusion that the cycle is probably ubiquitous among this group of animals (Krebs, 1954). There is also evidence for the operation of the cycle, or for one or more reactions of the cycle, in several groups of invertebrates which have been studied (*ibid;* Read, 1960). However, some of the trypanosomes lack the tricarboxylic acid cycle (von Brand, 1957) and this pathway is reported to be absent in *Trichomonas vaginalis* (Wirtschafter *et al.*, 1956; Read, 1957) although the closely related species, *T. gallinae*, possesses enzymes which oxidize the intermediates of the cycle (Read, 1957).

The present communication reports some studies which indicate that the vinegar eel (*Turbatrix aceti*), a free-living nematode, may lack the tricarboxylic acid cycle in its usual form.

Materials and Methods

Culture and preparation of vinegar eels

The vinegar eels used in these experiments were grown from cultures obtained from Turtox Biological Supply Company. The organisms were grown by inoculating commercial clear apple juice diluted 1:1 with water with a dense suspension of eels in vinegar (about 20 ml. per quart of diluted apple juice). Other fruit juices, *e.g.*, pear, peach, apricot, may also be used although the sediment interferes with observation and cleaning of the worms. The inoculated juice was poured into gallon bottles to a depth of about one inch. The bottles were covered loosely and kept at room temperature. After 7–10 days the fermenting cultures were dense with vinegar eels. At this point the bottles were half-filled with vinegar. These cultures could be kept for long periods of time with no attention. If the fermenting juice was kept too long without adding vinegar, putrefaction with loss of the culture often occurred. With a little experience it was possible to judge the

[1] Part of this work was carried out in the Zoology Department, University of California, Los Angeles, California, during the tenure (senior author) of a predoctoral Fellowship from the National Institutes of Health, U. S. Public Health Service, and constituted a portion of a thesis submitted to the Graduate Division in partial fulfillment of the requirements for the Ph.D. degree.

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right time for the addition of the vinegar. For some experiments reported the organisms were grown by inoculation into a medium containing peptone (Difco) 0.5%, yeast extract (Difco) 0.3%, NaCl 0.5%, and acetic acid 2–4%. The pH was about 3 to 3.5, depending on the amount of acetic acid added. This medium offers no advantages over vinegar, and the establishment of a dense culture requires several months.

Several methods of concentrating the organisms and washing to free them of the bulk of contaminating organisms were used. The following method, which was found to be the most satisfactory, depends on the negative geotropism of the vinegar eels. The cultures in vinegar or other medium are subjected to a preliminary concentration in a separatory funnel. Most of the organisms will, within a few minutes, aggregate at the surface of the liquid in a dense layer ¼ inch or so deep. The lower liquid is drained off rapidly. The concentrated suspension remaining is poured into burettes (50- or 100-ml.). After a few minutes the lower portion of the burette will contain few worms and the organisms will have begun to aggregate at the surface. The stopcock is opened slightly and the liquid allowed to drain out dropwise at a rate which will leave the surface-aggregated organisms behind, adhering to the wall of the burette. If the outflow rate is properly adjusted, most of the vinegar eels will remain on the walls of the burette when the liquid has drained out. The worms are rinsed from the burette with a small amount of a suitable solution (*e.g.*, 3% acetic acid or 0.05 *M* phthalate buffer, pH 3.0). When a sufficient number of worms has been collected they may be washed any desired number of times by repetition of this procedure or by brief centrifugation (500 × G). The latter procedure is not recommended except for the final washings and packing of the organisms, since repeated centrifugation damages the worms to the extent of interfering with their normal negative geotropic behavior.

For some of the experiments reported the organisms were prepared by washing them two or three times as above, holding them for 24 hours in a non-nutrient medium (0.05 *M* phthalate, pH 3.0), re-washing as above, and finally washing three times by centrifugation in the medium in which the organisms were to be finally suspended or homogenized.

In earlier experiments (the determination of substrate oxidation by intact organisms), where it was necessary to free the worms as completely as possible from contaminating micro-organisms, the vinegar eels were prepared by centrifuging two or three times with mechanical removal of the bulk of contaminating micro-organisms which form a layer above the denser eels. Following this initial washing, the suspension was placed in a Buchner-type funnel with a sintered glass disc of medium porosity through which 100–200 ml. of 0.05 *M* phthalate, pH 3.0, were allowed to run over a period of several hours. Final concentration of the organisms was achieved by centrifugation in all cases. Rapid attainment of the relatively low centrifugal force (about 500 × G) necessary for sedimentation of the organisms and rapid deceleration are essential for satisfactory sedimentation. The total time needed, from starting to stopping, is less than two minutes. Longer centrifugation injures the worms and slow stopping allows time for redispersion. The most satisfactory instrument for the purpose is a small table-top, swinging cup centrifuge. This type rapidly attains maximum speed and can be stopped quickly.

Determination of oxygen consumption

In a typical manometric experiment with intact *T. aceti*, 2.7-ml. portions of a dense suspension of organisms (giving a reading of about 100 units with a #54 green filter in the Klett-Summerson colorimeter against a water blank) in the appropriate buffer were pipetted into Warburg flasks of 12–18-ml. capacity. Folded filter paper and 0.2 ml. of 30% KOH were placed in the center wells. Substrate or inhibitor dissolved in buffer (0.3 ml.), to give the final concentration indicated, was pipetted into the side bulb. Following an equilibration period, readings of endogenous oxygen consumption were taken at 15-minute intervals for a two-hour period. Substrate (or inhibitor) was then added from the side bulb and the oxygen consumption measured during another two-hour period. Control flasks with buffer alone in the side bulb were included to correct for changes in endogenous oxygen consumption. In some of the experiments reported the side bulb contents were tipped in at the beginning of the measurement period. Experiments with intact organisms were carried out at 24.5° C. The gas phase was air. The system was buffered with 0.05 *M* phthalate except where otherwise noted. At the conclusion of an experiment the total nitrogen content of each flask was determined by the method of Johnson (Umbreit, Burris and Stauffer, 1949). Oxygen consumption is reported as μl. of oxygen consumed per mg. of tissue nitrogen per hour (Q_{O_2} (N)) except in the experiments with fluoracetate where the total oxygen consumed in 200 minutes is reported.

Enzyme assays

Homogenates were prepared in all-glass homogenizers immersed in ice water. Homogenates were prepared on the basis of the volume of packed vinegar eels following a two-minute centrifugation at about 500 × G. Thus a 5% homogenate was made by dispersing 0.2 ml. of packed organisms in 4 ml. of homogenizing medium. Homogenization performed intermittently for 5 to 8 minutes yielded a milky suspension almost free of intact worms. Homogenates for manometric measurement of succinic or malic dehydrogenase were prepared, in different experiments, in water, 0.154 *M* KCl or in 0.5 *M* sucrose. For the spectrophotometric assays using phenazine methosulfate as an electron carrier, the homogenates were prepared in 0.5 *M* sucrose. Homogenates for measurement of oxidation of keto acids were made in 0.154 *M* KCl containing 8 ml. of 0.04 *M* $KHCO_3$ per liter.

Succinic dehydrogenase was assayed in homogenates by three methods. (1) The manometric method of Schneider and Potter (Umbreit, Burris and Stauffer, 1949). (2) The Thunberg method: the system contained 0.03 *M* sodium phosphate, 0.045 *M* sodium succinate, 0.05 mg./ml. methylene blue, pH 7.4, total volume 5.0 ml. After evacuation and equilibration at 37° C., one ml. of a 10% *T. aceti* homogenate in water was added from the side bulb of the Thunberg tube. At intervals during a two-hour period at 37° the optical density of the tubes was measured at 600 mμ. (3) A colorimetric method employing phenazine methosulfate (PMS) to mediate electron transfer between the enzyme and dichloroindophenol (Ells, 1959). The assay system contained 0.05 *M* potassium phosphate, 0.01 *M* KCN, 2.3×10^{-5} *M* sodium 2,6-dichloroindophenol, 0.02 mg. PMS, and 0.01 *M* sodium succinate. The pH of the system was 7.4 and the total volume was 6.0 ml.

The change in the optical density of the system was followed spectrophotometrically at 610 mμ. Succinate oxidized was assumed to be equal, on a molar basis, to dichloroindophenol reduced.

Malic dehydrogenase was tested for by the method of Potter (Umbreit, Burris and Stauffer, 1949) and by an adaptation of the colorimetric PMS method. The reaction mixture for the latter assay was the same as the system for succinic dehydrogenase except that 3.3×10^{-4} *M* pyridine nucleotide (DPN or TPN) was added and 0.008 *M* sodium DL-malate was substituted for succinate. Sodium fumarate (0.0067 *M*) was added in addition to or in place of malate in some assays.

TABLE I

Effect of 0.05 M acetate on respiration of intact T. aceti. The pH of the system was 3.0. Time refers to the time lapse between removal of the organisms from the original culture and beginning of the measurement of respiration. Figures starred are averages of duplicates, the others of triplicates.

Time (hours)	$Q_{O_2}(N)$		% Change
	Endogenous	With acetate	
3	142	189	+33
24	72*	335*	+365
39	62	136	+119

Attempts were made to demonstrate isocitric dehydrogenase in *T. aceti* homogenates in a system containing 0.05 *M* Tris, 0.04 *M* sodium DL-isocitrate, 0.001 *M* $MnSO_4$, 2.3×10^{-5} *M* sodium 2,6-dichloroindophenol, 0.02 mg. phenazine methosulfate and either 6.7×10^{-4} *M* each of DPN and AMP or 6.7×10^{-4} *M* TPN. The pH was 7.4. The optical density at 610 mμ was measured at 30-second intervals for at least 10 minutes in all experiments.

The oxidation of α-keto acids (pyruvic, oxalacetic and α-ketoglutaric) was measured manometrically by the method of Potter, Pardee and Lyle (Umbreit, Burris and Stauffer, 1949).

Citrate synthesis was measured following incubation of a suspension of intact vinegar eels at room temperature in 0.02 *M* sodium acetate, pH 4.5. At the end of the incubation period 1.0-ml. aliquots of the suspension were rapidly homogenized and deproteinized by the addition of 1 ml. of 5% sodium tungstate and 1 ml. of 0.33 *M* H_2SO_4. Citrate was determined in the filtrates by the method of Taussky and Shorr (1947).

RESULTS

Endogenous oxygen consumption and oxidation of acetate

Suspensions of vinegar eels in phthalate or phosphate buffer show $Q_{O_2}(N)$ values of about 50 to 200, depending on the time lapse between removal of the organisms from a nutrient culture medium and measurement of oxygen consumption. The upper value is approached by organisms used within two or three hours of removal from culture while the lower values are obtained with organisms maintained in a non-nutrient solution for 36 hours or more.

Acetate markedly stimulates the respiration of vinegar eels, as shown by the data in Table I. Following a three-hour period without exogenous substrate, while the endogenous respiration is still high, acetate produces only a slight stimulation of oxygen consumption. As the endogenous respiration falls, the stimulatory effect

TABLE II

Effect of Krebs cycle intermediates on the respiration of intact T. aceti. Respiration was measured at the pH indicated as described in the Methods section. "Before" and "after" refer to respiration before and after tipping in the contents of the side bulb (buffer alone or buffer with substrate), time as in Table I. In some experiments side bulb contents were tipped into flasks at the beginning of the measurement period. Figures starred are averages of duplicates, others of single determinations.

Substrate in side bulb	Final conc. (*M*)	Time (hrs.)	$Q_{O_2}(N)$								
			pH 3.0			pH 4.5			pH 6.0		
			Before	After	% Chg.	Before	After	% Chg.	Before	After	% Chg.
None	—	4	—	164*	—	—	—	—	—	—	—
Citrate	0.05	4	—	167*	—	—	—	—	—	—	—
None	—	8	—	232*	—	—	—	—	—	—	—
Citrate	0.05	8	—	247*	—	—	—	—	—	—	—
None	—	23	100	89	−11	80	74	−8	82	99	+21
Citrate	0.05	23	125*	117*	−7	96*	89*	−7	93*	93*	0
None	—	48	66	81	+23	70	81	+16	63	79	+25
Citrate	0.01	48	74*	81*	+9	65*	81*	+25	61*	82*	+34
None	—	4	—	164*	—	—	—	—	—	—	—
Succinate	0.05	4	—	191*	—	—	—	—	—	—	—
None	—	8	—	232*	—	—	—	—	—	—	—
Succinate	0.05	8	—	210*	—	—	—	—	—	—	—
Succinate	0.05	17	96*	80*	−10	126*	123*	−2	120*	107*	−11
None	—	48	51	67	+31	93	93	0	45	68	+51
Succinate	0.01	48	70*	89*	+27	85*	85*	0	57*	79*	+39
None	—	4	—	164*	—	—	—	—	—	—	—
DL-malate	0.05	4	—	100*	—	—	—	—	—	—	—
None	—	8	—	232*	—	—	—	—	—	—	—
DL-malate	0.005	8	—	216*	—	—	—	—	—	—	—
None	—	23	100	89	−11	80	74	−8	82	99	+21
DL-malate	0.05	23	104*	107*	+3	104*	97*	−7	98*	96*	−2
None	—	48	66	81	+23	70	81	+16	63	79	+25
DL-malate	0.01	48	85*	98*	+15	81*	94*	+16	69*	95*	+38
None	—	16	—	—	—	59*	73*	+24	74*	86*	+16
Oxalacetate	0.05	16	50*	108*	+116	71*	121*	+72	97*	143*	+47

TABLE III

Effect of malonate on respiration of intact T. aceti. Respiration was measured at pH 4.5. Figures starred are averages of duplicates, the others of single determinations.

Acetate (*M*)	Malonate (*M*)	$Q_{O_2}(N)$		% Change
		Before addition	After addition	
0	0.001	65*	89*	+37
0	0.02	71	103	+45
0.01	0	53*	116*	+119
0.01	0.001	65*	130*	+100
0.01	0.02	61*	149*	+144
0.05	0	48	166	+246
0.05	0.001	46*	175*	+280
0.05	0.02	57*	170*	+198

of acetate increases. The explanation for the very much higher acetate stimulation in worms which had been maintained in non-nutrient medium for 24 hours than in worms three hours out of the original culture is not known. This kind of effect was fairly regularly observed although it was not always so striking. The lower effect of acetate on the respiration of organisms which have been substrate-depleted for much longer periods of time (39 hours in Table I) is also characteristic and perhaps reflects irreversible damage to the metabolic machinery.

Effect of Krebs cycle intermediates on respiration of intact organisms

Several intermediates of the Krebs cycle were added to suspensions of intact *T. aceti*, usually after preliminary measurement of endogenous respiration. In some experiments the endogenous respiration and the respiration in the presence of substrate were measured simultaneously in different flasks. Usually two substrate concentrations were used and organisms exhibiting two or more levels of endogenous respiration were tested with each substrate. Each substrate was tested at pH 3.0, 4.5 and 6.0. The results are shown in Table II. Under the conditions employed, citrate, succinate and malate appear to be without significant effect on oxygen consumption compared with the control flasks in which buffer without substrate was added from the side bulb. Oxalacetate, however, does stimulate respiration. The increased effect of this compound at lower pH is probably due to

TABLE IV

Effect of fluoracetate on the respiration of intact T. aceti. Respiration was measured in 0.05 M phosphate with 0.05 M acetate, pH 7.3. In each experiment the suspension of organisms was adjusted to give a reading of 130 units in the Klett-Summerson colorimeter with a #54 filter.

μl./200 minutes	
No fluoracetate	0.01 *M* fluoracetate
52.9	27.1
73.2	37.7
57.6	29.8

more rapid penetration of the organisms by undissociated oxalacetic acid than by oxalacetate ion.

Effect of malonate on respiration of intact organisms

Table III summarizes data on the effect of malonate on the respiration of intact *T. aceti*. Endogenous oxygen consumption is not inhibited by malonate at concentrations of 0.001 and 0.02 *M*, and there may be a slight stimulation of respiration although the number of determinations is too small to establish this with certainty.

Citrate synthesis and the effect of fluoracetate

Fluoracetate (0.01 *M*) was found to inhibit the oxygen consumption of intact vinegar eels, as shown in Table IV. This suggested that a pathway for the synthesis of citrate and aconitase, or some other fluoracetate-sensitive enzyme catalyzing the further metabolism of citrate might be operative in these organisms. Intact

TABLE V

Synthesis of citrate by T. aceti in the presence and in the absence of fluoracetate. Incubations performed in 0.02 M sodium acetate, pH 4.5.

Experiment	Incubation period	Citrate synthesized (μg./mg. (N))	
		No fluoracetate	0.01 *M* fluoracetate
1	30 mins.	11.0	11.0
2	2 hrs.	12.4	41.2
3	8 hrs.	30.2	176.0
4	8 hrs.	93.4	181.3

T. aceti which had been maintained several hours in a non-nutrient medium were found to synthesize citrate when acetate was added to the medium (Table V). Furthermore, the accumulation of citrate under these conditions was increased in the presence of 0.01 *M* fluoracetate.

Substrate oxidation by T. aceti homogenates

Several attempts were made, without success, to demonstrate succinic dehydrogenase manometrically in homogenates of vinegar eels fortified with cofactors necessary for respiration by homogenates of mammalian tissues. The reaction mixture included mammalian cytochrome *c* to serve as an electron carrier. Rat liver homogenate controls consumed oxygen rapidly in this system. Experiments using the Thunberg technique showed that *T. aceti* homogenates did not reduce methylene blue anaerobically in the presence of succinate.

Neither cytochrome *c* nor methylene blue can accept electrons directly from mammalian succinic dehydrogenase (Singer and Kearney, 1954) but only from other electron carriers which are able to react with the enzyme. Phenazine methosulfate is the most active artificial electron acceptor which will react directly with succinic dehydrogenase. When this dye was used, with dichloroindophenol as the

TABLE VI

Oxidation of succinate by T. aceti homogenate using phenazine methosulfate as an electron carrier

System	Succinate oxidized (mμmoles/min./ml. 5% homogenate)	
	Experiment 1	Experiment 2
Complete	7.6	5.5
Minus succinate	0.6	0.0

terminal electron acceptor, a low succinic dehydrogenase activity was demonstrated in vinegar eel homogenates (Table VI).

T. aceti homogenates containing DPN, cytochrome *c* and other cofactors necessary for the demonstration of malic dehydrogenase in mammalian tissues did not consume oxygen in the presence of malate. Rat liver homogenate controls showed a high activity. When PMS was used to couple electron transfer between reduced pyridine nucleotides and dichloroindophenol, malate was rapidly oxidized by *T. aceti* homogenates in the presence of DPN (Table VII). TPN would not replace DPN, and fumarate at a concentration nearly equal to that of the DL-malate in the system did not inhibit. When fumarate was used as substrate in this system instead of malate, the rate of reduction of dichloroindophenol was less than 10% of that with malate, indicating that the fumarase activity is very low.

There was no oxygen consumption by vinegar eel homogenates in the presence of pyruvate, oxalacetate or α-ketoglutarate and the cofactors necessary for the oxidation of these keto acids by mammalian tissue homogenates. Isocitric dehydrogenase activity could not be demonstrated in *T. aceti* homogenates using either TPN and Mn^{++} or DPN and AMP as cofactors. PMS, which will accept electrons from either DPNH or TPNH, was used as an electron carrier in these experiments.

DISCUSSION

The experiments with homogenates, together with the finding that several of the Krebs cycle intermediates do not stimulate the respiration of intact *T. aceti*, suggests that the cycle, in its usual form, does not operate in this species. Stimula-

TABLE VII

Oxidation of malate and fumarate by T. aceti homogenate using phenazine methosulfate as an electron carrier

Substrate	Pyridine nucleotide	Substrate oxidized (mμmoles/min./ml. of 5% homogenate)	
		Experiment 1	Experiment 2
DL-malate	DPN	152	140
Fumarate + DL-malate	DPN	—	144
Fumarate	DPN	8.2	13.0
None	DPN	0	—
DL-malate	None	2.4	—
DL-malate	TPN	4.7	3.5
DL-malate (no PMS)	DPN	9.4	—

tion of the respiration of intact worms by oxalacetate, demonstration of citrate synthesis, and the inhibition of respiration by fluoracetate indicate that citrate synthetase and aconitase may be present. Isocitric dehydrogenase is apparently absent and the activities of succinic dehydrogenase and fumarase are so low under the conditions of the assay (compared with malic dehydrogenase) as to raise doubt that they function in the main pathway of oxidative metabolism. Indeed, it is not certain that these activities are not due to unavoidable contamination of the homogenates with micro-organisms, although this seems doubtful. Failure of homogenates to oxidize the keto acids under the conditions employed is not conclusive evidence for the inertness of these compounds in *T. aceti* since PMS was not used as electron carrier in these systems.

In the experiments with intact vinegar eels the possibility that a high endogenous respiration might mask the effect of oxidizable Krebs cycle intermediates has been obviated by using organisms at several time intervals after removal from a nutrient medium. The effect on intact organisms of utilizable substrates might be thought to depend on the pH at which measurements are made, as has been shown with *Euglena* (Danforth, 1953). However, in the present study changes in pH over the range 3.0 to 6.0 have no effect on respiration in the presence of the compounds tested, with the exception of oxalacetate. Failure of malate to stimulate the respiration of intact vinegar eels under these conditions is difficult to understand in view of the active malic dehydrogenase these organisms possess. It may be that intact *T. aceti* are impermeable to some organic acids at all pH values. This possibility admittedly casts doubt on conclusions concerning the absence of metabolic reactions based on the failure of compounds to stimulate respiration of intact organisms of this species.

The finding that malonate has no inhibitory effect on acetate oxidation by intact vinegar eels and slightly increases the endogenous respiration may further confirm the thesis that the main pathway of acetate oxidation does not go through succinic dehydrogenase. However, this is not conclusive evidence because of the above-mentioned permeability problems. Read (1956) showed a slight stimulation of the respiration of intact *Hymenolepis diminuta* by malonate, although in cell-free preparations malonate inhibited the oxidation of succinate.

It is clear from the experiments with homogenates, and especially strikingly illustrated in the case of the malic dehydrogenase experiments, that *T. aceti* cannot use mammalian cytochrome *c* in electron transport. Lack of cytochrome *c* reductase or cytochrome oxidase may explain this. Studies of the electron transport system in *T. aceti*, to be published elsewhere, indicate that the latter enzyme, at least, is absent.

Ascaris, a nematode whose oxidative metabolism has been studied in some detail, shows similarities to *T. aceti*. Although a number of intermediates of the citric acid cycle fail to stimulate the respiration of intact *Ascaris* (Rathbone, 1955), fumarase and malic decarboxylase are present (Saz and Hubbard, 1957) and a succinic dehydrogenase has been partially purified (Bueding *et al.*, 1955). Cytochrome *c* and cytochrome oxidase are absent (Bueding and Charms, 1952; Rathbone, 1955). The malic decarboxylase, which oxidatively decarboxylates L-malate to pyruvate, requires either DPN or TPN, the latter being less active (Saz and Hubbard, 1957).

The role of the malic dehydrogenase in *T. aceti* remains in doubt pending further study. It is possible that this enzyme will prove to be a decarboxylase.

Summary

1. Suspensions of intact *Turbatrix aceti* in phthalate or phosphate buffer consume oxygen at a rate varying from about 50 to 200 μl./mg. (tissue nitrogen)/hr. The rate depends on the time lapse between removal of the organisms from the original culture and the beginning of the measurement, *i.e.*, the time without exogenous substrate.

2. Oxygen consumption of intact organisms is markedly stimulated by acetate and also by oxalacetate.

3. Citrate, succinate and malate do not stimulate the oxygen consumption of intact *T. aceti* at pHs 3.0, 4.5 or 6.0 and when tested with organisms showing several levels of endogenous respiration.

4. Malonate at a concentration of 0.001 *M* or 0.02 *M* does not inhibit the endogenous oxygen consumption of intact *T. aceti* nor does it prevent the stimulation of respiration by acetate.

5. Citrate is synthesized by intact organisms in the presence of acetate. Fluoracetate increases this synthesis and inhibits the respiration of intact vinegar eels.

6. Pyruvate, α-ketoglutarate, succinate, malate and oxalacetate are not oxidized by homogenates of *T. aceti* fortified with cofactors necessary for oxidation of these compounds by mammalian liver preparations.

7. When phenazine methosulfate is used to transfer electrons from pyridine-nucleotides to dichloroindophenol, an active DPN-malic dehydrogenase can be demonstrated in homogenates. TPN has little or no activity. When fumarate is used in place of malate in this system, activity is low, indicating a low fumarase activity.

8. Using phenazine methosulfate to accept electrons from the enzyme, with dichloroindophenol as terminal electron acceptor, a low succinic dehydrogenase activity can be shown in *T. aceti* homogenates.

9. Isocitric dehydrogenase was not detected in a system containing phenazine methosulfate and dichloroindophenol as electron acceptors and either TPN and Mn^{++} ions or DPN and AMP as cofactors.

10. These findings suggest that, in addition to the active malic dehydrogenase, *T. aceti* possesses citrate synthetase and aconitase but apparently has limited ability to carry out other reactions of the tricarboxylic acid cycle. The electron transport system, unlike that of many invertebrates, cannot utilize mammalian cytochrome *c*.

11. If these interpretations are borne out, *T. aceti* will be the first animal with a predominantly aerobic metabolism in which the tricarboxylic acid cycle in its usual form has been shown to be absent.

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PHYSIOLOGY OF THE MELANOPHORES IN THE CRAB SESARMA RETICULATUM[1]

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In 1948 Brown noted that the black pigment in all crabs, which had been observed, concentrated maximally after eyestalk removal if the animals were not exposed to excessively high intensities of illumination. Later, however, Enami (1951) reported that the pigment in melanophores of specimens of *Sesarma intermedia, S. haematocheir,* and *S. dehaani* dispersed maximally after eyestalk ablation.

Regulation of color change has been studied in a detailed manner in very few species of crabs. In the fiddler crab, *Uca pugilator,* the species whose chromatophores have been studied most intensively, the melanophores are controlled by pigment-dispersing and pigment-concentrating substances (Carlson, 1936; Sandeen, 1950; Brown and Fingerman, 1951; Fingerman, 1956a). A black-pigment-dispersing principle has been found also in the crabs *Hemigrapsus oregonensis* by Bowman (1949), *Callinectes sapidus,* the blue crab, by Fingerman (1956b), *Eriocheir japonicus* by Matsumoto (1954), *Uca rapax* by Burgers (1958), and *Macropipus vernalis* by Burgers (1959). No evidence, however, has been adduced for a melanin-concentrating principle in these crabs. Enami (1951) reported that extracts of central nervous organs from the three species of *Sesarma* evoked concentration of melanin when injected into eyestalkless individuals.

The responses of melanophores to changes in light intensity and temperature have been observed in *Uca pugilator* (Brown and Sandeen, 1948) and *Callinectes sapidus* (Fingerman, 1956b). An albedo response operated to disperse the black pigment when specimens of both species were placed on a black background and to concentrate this pigment in crabs on a white background. Enami (1951) stated that background responses were not exhibited by the three species of *Sesarma* which he observed. With respect to temperature, the melanin in *Uca* tended to concentrate as the temperature increased above or decreased below 15° C. In contrast, the black pigment in *Callinectes* became progressively more concentrated as the temperature increased from 10° to 28° C.

The present investigation was undertaken primarily to expand the small amount of information available concerning chromatophores of brachyurans other than *Uca,* and secondarily to compare the behavior of the melanophores in specimens of *Sesarma reticulatum* from the Woods Hole area with the findings reported by Enami (1951) for three species of *Sesarma* in Japan. Problems such as the chromatics of eyestalkless specimens, background responses, and endocrine regulation of the melanophores were considered.

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Materials and Methods

Adult specimens of the grapsoid crab *Sesarma reticulatum* were collected at West Falmouth, Massachusetts, for use in this investigation. In the laboratory crabs were maintained under constant illumination in aquaria containing sea water approximately 2 cm. deep. The intensity of illumination incident on these crabs was 40 ft. c.

Melanophores on the walking legs were observed with the aid of a stereoscopic dissecting microscope and transmitted light and were staged according to the system of Hogben and Slome (1931). Stage 1 represented maximal concentration of the melanin, stage 5 maximal dispersion, and stages 2, 3 and 4 the intermediate conditions.

Tissue extracts were prepared in sea water as described in detail by Sandeen (1950). The dose of each extract injected into assay animals was 0.05 ml. The concentration of each extract was one-third of a complement per 0.05 ml. Eyestalkless specimens, some of which served as assay animals, had had both eyestalks removed at least 12 hours prior to use. The wounds were cauterized to minimize loss of blood.

Student's t test was used for determination of the level of significance between the means. The 5% level was considered the maximum for a significant difference. Standard deviations and standard errors for the differences between means were also calculated.

Experiments and Results

Rhythm of pigment migration

Twenty intact specimens were placed into a darkroom during the afternoon of July 19, 1960. At midnight the average stage of the melanophores of 10 specimens was determined and the crabs were returned to the darkroom. The same procedure was followed at 8:00–8:30 AM, noon–1:00 PM, 5:00 PM, and midnight Eastern Daylight Saving Time for the next seven days.

The means are presented in Figure 1. Inspection of this figure reveals that the melanin was more dispersed by day than at night. The chromatophore indices determined at midnight and at 8:00–8:30 AM for the entire series of observations were compared to determine the statistical significance of the difference between the means. This difference was highly significant.

Relationship between light intensity, background, and chromatophore stage

The objectives of this set of experiments were to determine during the day phase and the night phase of the cycle of pigment migration (1) if specimens of *Sesarma reticulatum* exhibit a background response and (2) the response of the melanophores to changes in total intensity of illumination. For the first experiment 10 intact, recently collected specimens were placed into each of six black and six white pans at 8:00 AM. The animals in one black and one white container then were exposed to one of these intensities of illumination, 2, 17, 40, 70, 250, and 1110 ft. c., for two hours at which time the melanophores of each crab in the 12 containers were staged. This experiment was repeated on another day with one change in the procedure; the crabs were placed under the various intensities at noon instead of at 8:00 AM.

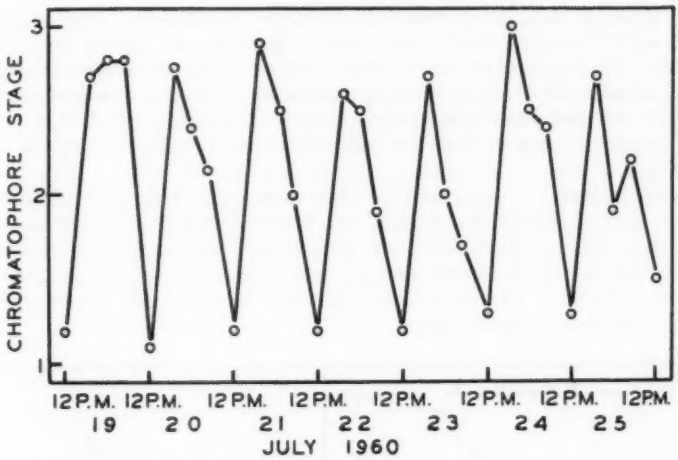

FIGURE 1. Relationship between melanophore stage and time of day in crabs maintained in darkness.

The next two experiments were performed at night between 10:45 PM and 3:00 AM. Ten intact crabs were placed into each of four black and four white pans. The crabs in one black and one white container were then exposed to one of these light intensities, 2, 32, 280, and 1110 ft. c., for two hours. The chromatophore stage of each crab was then determined. This experiment was also performed one more time.

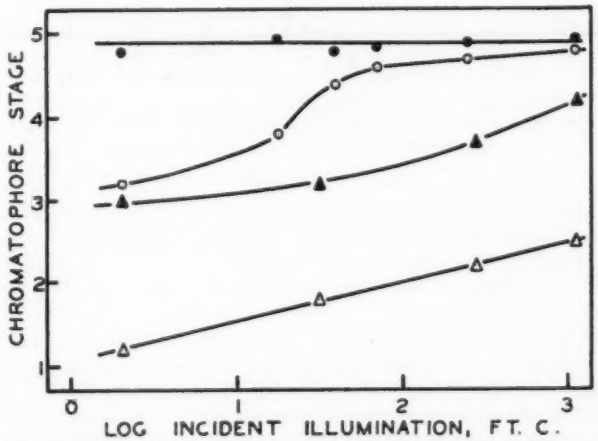

FIGURE 2. Relationships between melanophore stage and the logarithm of the incident light intensity for crabs in the day phase of the rhythm and on a black background (dots), day phase and on a white background (circles), night phase amd on a black background (solid triangles), night phase and on a white background (empty triangles).

The means of the data obtained from these experiments were used in the preparation of Figure 2. Inspection of this figure reveals several facts. During the daytime the pigment in the melanophores of crabs in black containers was nearly maximally dispersed at each light intensity used in the experiments. For example, the mean chromatophore stages were 4.75 at 2 ft. c. and 4.95 at 1110 ft. c. Statistical analysis revealed that the difference between these means was not significant.

The melanophores of the crabs in white containers, however, behaved differently. At 2 ft. c. during the daytime the pigment was in an intermediate state (mean stage, 3.15). With increased illumination the degree of pigment dispersion increased with the result that the mean chromatophore stage of the crabs exposed to 1110 ft. c. was 4.80. The difference between these means (3.15 and 4.80) was statistically significant.

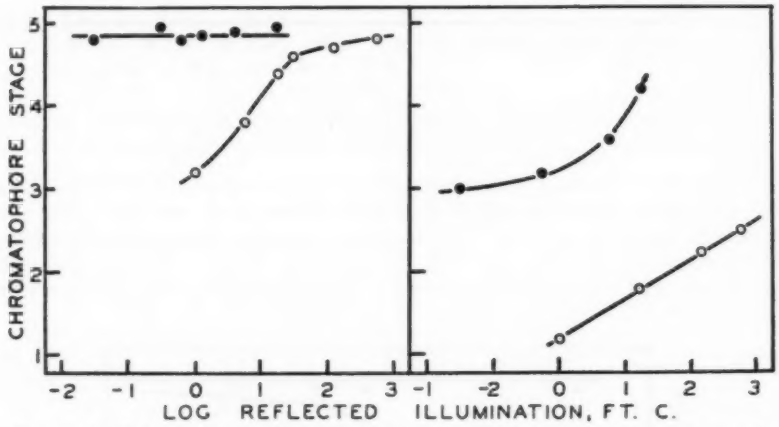

FIGURE 3. Relationships between melanophore stage and the logarithm of the reflected light intensity for crabs in the day phase (left portion of the figure) and night phase (right portion of the figure) of the rhythm. Circles, on a white background; dots, on a black background.

With regard to the experiments performed at night, crabs in the black and in the white pans darkened in response to increased illumination. The increased dispersion of pigment with increased light intensity in crabs on both backgrounds was statistically significant. The data obtained at 2 and 1110 ft. c. were used in these analyses.

Inspection of Figure 2 reveals that, in addition to the response to incident illumination, the melanin was more dispersed in crabs on a black background than on a white one, with the possible exception of the crabs exposed to 1110 ft. c. by day. The difference between the mean chromatophore stages of the crabs on the black and the white backgrounds during the daytime at an illumination of 2 ft. c. was statistically significant but not at 1110 ft. c. However, the differences between the crabs on the two backgrounds during the nighttime were significant at both 2 and 1110 ft. c.

The findings presented in Figure 2 bear out the cycle of pigment migration shown in Figure 1. At each light intensity used the pigment of crabs on both backgrounds was more dispersed by day than at night. Statistical analysis revealed that the differences between chromatophore stages obtained at 2 and 1110 ft. c. during the nighttime and daytime were highly significant.

To demonstrate clearly that specimens of *Sesarma reticulatum* show a true background (*i.e.*, albedo) response and not merely a response to the amount of light reflected from the background, the data of Figure 2 were replotted in terms of the intensity of light reflected from the black and the white backgrounds (Fig. 3). The white background reflected one-half the incident illumination and the black background 1/60. The existence of an albedo response was evident be-

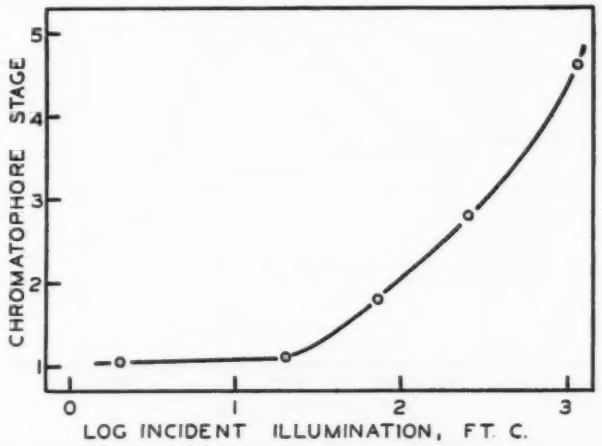

FIGURE 4. Relationship between melanophore index of eyestalkless specimens of *Sesarma reticulatum* on a white background and the logarithm of the incident light intensity.

cause the data obtained on the black and the white backgrounds did not overlap although the light intensity had increased 505-fold whereas the white background reflected only 30 times more light than did the black background.

The next experiment was designed to determine the relationship between illumination and the degree of pigment dispersion in the melanophores of eyestalkless specimens of *Sesarma reticulatum*. Eyestalkless specimens in white pans were exposed between 8:20 and 10:20 AM to the following illuminations: 2, 20, 70, 250, and 1110 ft. c. The melanin was maximally concentrated in crabs exposed to 2 ft. c. and was nearly maximally dispersed in crabs at 1110 ft. c. This experiment was repeated. Means were used in the preparation of Figure 4. The degree of pigment dispersion in eyestalkless specimens can be readily altered by changing the intensity of incident illumination. This pigment migration is presumably a direct response of the melanophores to light. The difference between the means at 2 and 1110 ft. c. was statistically significant.

Relationship between temperature and melanophore stage

The aim of this set of experiments was to determine whether the melanophores of *Sesarma reticulatum* are sensitive to temperature changes. During the daytime 10 intact crabs were placed into each of five white pans which contained sufficient sea water to cover the crabs. These crabs were then exposed to an illumination of 2 ft. c. The white background, daytime, and light intensity of 2 ft. c. were chosen because under these conditions the melanophores of the crabs were at approximately stage 3 (Fig. 2). Through use of water baths each pan was exposed for two hours to one of the following temperatures: 3.5°, 10°, 22.5°, 30°, and 36° C. The average stage of the melanophores of each crab was determined at the end of the exposure period. This experiment was performed twice.

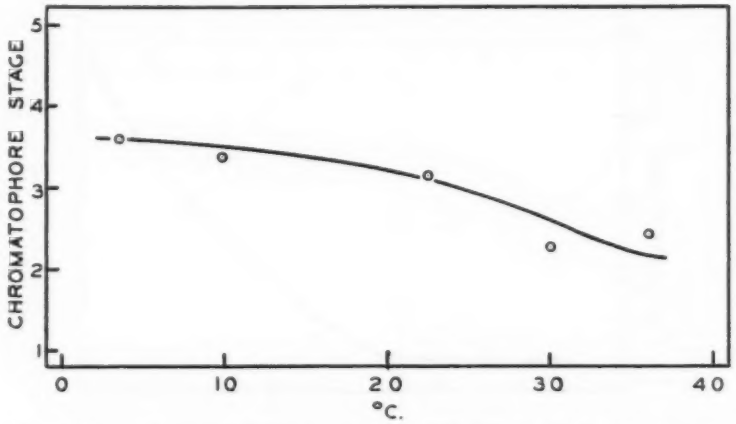

FIGURE 5. Relationship between temperature and melanophore index of intact specimens on a white background under an incident illumination of 2 ft. c. during the daytime.

An inverse relationship between temperature and chromatophore stage was apparent (Fig. 5). For purposes of statistical analysis the data obtained at (1) 3.5° and 10° C., and (2) 30° and 36° C. were grouped. The difference between the means of these two groups of data was statistically significant.

Endocrine regulation of the melanophores

The object of the final set of experiments was to determine the role of endocrines in mediating pigment migration in the melanophores of *Sesarma reticulatum*. For use in the first set of experiments of this group extracts of the optic ganglia, supraesophageal ganglia, circumesophageal connectives, and thoracic ganglia were prepared in the manner described earlier under Materials and Methods. Each extract was injected into five eyestalkless specimens in a white pan under an illumination of 2 ft. c. Under these conditions the melanin was maximally concentrated. The control consisted of eyestalkless crabs which received sea water injections. Each extract dispersed the black pigment.

This experiment was repeated twice. The data of the three experiments were averaged and the means were used in the preparation of the upper portion of Figure 6. The amount of pigment dispersion produced by each extract was highly significant.

The next experiment was similar to the previous one with the single change that the crabs were exposed to an illumination of 560 ft. c. Under this light intensity the pigment was in an intermediate degree of dispersion (see Fig. 4). Substances that concentrate as well as disperse pigment can sometimes be detected

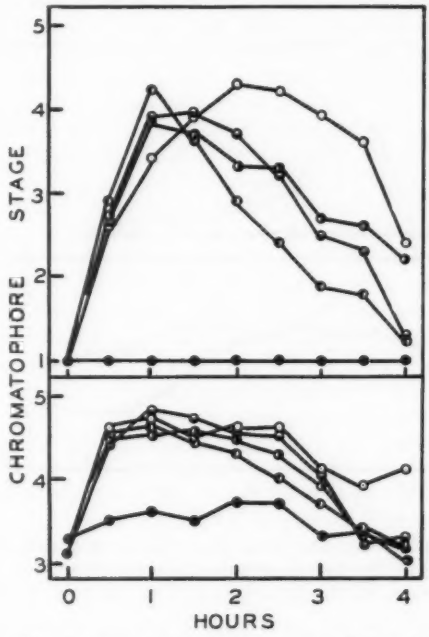

FIGURE 6. Relationships between melanophore stages and time following injection of extracts of central nervous organs into eyestalkless specimens in white containers under an illumination of 2 ft. c. (upper portion of figure) and 560 ft. c. (lower portion of figure). Optic ganglia, circles; supraesophageal ganglia, circles half-filled on bottom; circumesophageal connectives, circles half-filled on left; thoracic ganglia, circles half-filled on right; control, dots.

when the pigment of assay animals is in an intermediate state (Fingerman and Lowe, 1958). A pigment-dispersing substance was apparent but not a pigment-concentrating one. The experiment was repeated twice. The averaged data of the three experiments are shown in the lower portion of Figure 6. Each extract produced a statistically significant amount of pigment dispersion as was the case when extracts of these organs were injected into specimens with maximally concentrated melanin.

The remaining experiments involved assay of extracts prepared from sinus glands. Each extract was injected first into five eyestalkless crabs with maximally

concentrated black pigment and pigment dispersion was noted. This experiment was repeated twice with the same results. The means were used in the preparation of the upper portion of Figure 7 where each point represents 15 crabs. The amount of pigment dispersion was statistically significant. Controls consisted of eyestalkless specimens which received sea water injections.

When an extract of sinus glands was injected into eyestalkless crabs whose black pigment was in an intermediate stage of dispersion as a result of exposure to an illumination of 560 ft. c., a transitory increase in the degree of pigment dispersion occurred. This was followed by migration of the pigment to a more

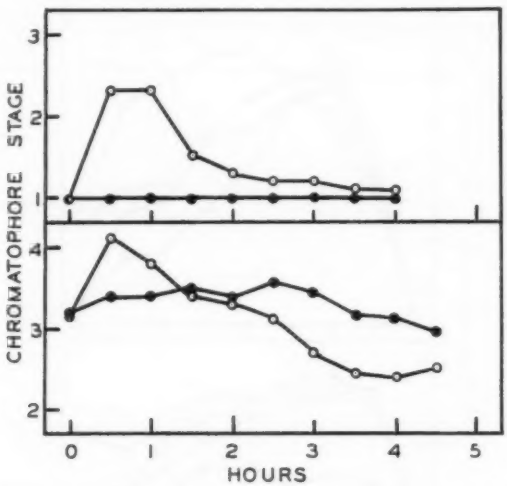

FIGURE 7. Relationships between melanophore stages and time following injection of extracts of sinus glands into eyestalkless specimens in white containers under an illuminatoin of 2 ft. c. (upper portion of figure) and 560 ft. c. (lower portion of figure). Sinus glands, circles; control, dots.

concentrated level than observed in the control group. This experiment was repeated three times. Means are presented in the lower portion of Figure 7 where each point represents 20 crabs. Statistical analysis of these data revealed that the amounts of both dispersion and concentration of melanin were statistically significant.

DISCUSSION

Specimens of *Sesarma reticulatum* showed a rhythm of color change which was manifested by a pale phase at night and a dark phase during the daytime (Fig. 1). Similar cycles have been observed in the fiddler crab, *Uca pugilator*, by Abramowitz (1937), in the blue crab, *Callinectes sapidus*, by Fingerman (1955), and in the swimming crab, *Macropipus vernalis* by Burgers (1959). Enami (1951) did not report on the possibility of a rhythm of color change occurring in the three species of *Sesarma* he used.

The responses to increased illumination of the melanophores in *Sesarma reticulatum, Uca pugilator* (Brown and Sandeen, 1948), and *Callinectes sapidus* (Fingerman, 1956b) are qualitatively alike. In intact specimens of these three crabs increased total illumination resulted in greater dispersion of the melanophore pigment. Enami (1951) stated that the three species of *Sesarma* he used exhibited a direct response to illumination, but he did not indicate the direction of the response. He also reported that these three species of *Sesarma* did not show a background response. However, specimens of *Uca pugilator, Callinectes sapidus,* and *Sesarma reticulatum* showed, in addition to the response to total illumination, an albedo response which called for more dispersion of melanin in crabs on a black background than on a white one. An interesting point concerning *Uca pugilator* is that in spite of the albedo response, at least during the daytime, the melanin of specimens on a white background was more dispersed than in crabs on a black background because the response to total illumination was stronger than the albedo response (Brown and Sandeen, 1948). In blue crabs, however, the albedo response was stronger than the response to total illumination (Fingerman, 1956b).

The amplitude of the response of melanophores in eyestalkless individuals of *Sesarma reticulatum* to total illumination (Fig. 4) appears to be unique among crustaceans. Maximal concentration of melanin is the typical response of brachyurans to eyestalk ablation if the crabs are not exposed to excessively high intensities of illumination (Brown, 1948). This response has been observed in a variety of crabs such as *Uca* (Carlson, 1936), *Hemigrapsus* (Bowman, 1949), and *Callinectes* (Fingerman, 1956b). As mentioned above, Enami (1951) observed three species of *Sesarma* in which the melanin dispersed maximally after eyestalk ablation instead of concentrating. Unfortunately Enami did not state the light intensity to which his crabs were exposed. At the light intensity usually recorded, about 30 ft. c., on the table tops of a laboratory, the melanin of *Sesarma reticulatum* was nearly maximally concentrated (Fig. 4), so that one would be inclined to conclude that the chromatic behavior of eyestalkless specimens of this species is more similar to that of *Uca* than to the behavior of the *Sesarma* studied by Enami. When eyestalkless *Uca* were exposed to an incident illumination as high as 3500 ft. c. (Brown and Sandeen, 1948), the black pigment dispersed only to an intermediate condition. The response of the melanophores in *Callinectes* to total illumination (Fingerman, 1956b) was not as great as was observed in *Uca* by Brown and Sandeen (1948).

In response to a rise in temperature, the black pigment of *Sesarma reticulatum* concentrated significantly (Fig. 5). This was also the response shown by the melanophores of *Callinectes* (Fingerman, 1956b). Such behavior suggested a thermoregulatory function of the black pigment. Crabs lightened at high temperatures and thereby reflected more heat from the body surface. Enami (1951) stated that the *Sesarma* he used showed a response to temperature but he did not define the nature of this response.

Extracts of central nervous organs from *Sesarma reticulatum* caused dispersion but no concentration of the pigment in melanophores (Fig. 6). However, extracts of the sinus glands caused melanin dispersion which was followed by a significant degree of melanin concentration (Fig. 7). Enami (1951) found that extracts of sinus glands and central nervous organs concentrated pigment when injected into eyestalkless specimens of *Sesarma intermedia, S. haematocheir,* and *S. dehaani.*

His experimental procedure did not allow assay for melanin-dispersing hormone, if this substance does exist in the crabs he used. The species of *Sesarma* used by Enami are not only intriguing with respect to the dispersed condition of the pigment in the melanophores of eyestalkless specimens but also with respect to the fact that the immediate effect of extracts of sinus glands from crabs investigated by Enami was concentration of pigment. Extracts of sinus glands from crabs utilized by the other investigators mentioned above always produced some dispersion of pigment.

It is interesting to note that in the assays of sinus glands of *Sesarma reticulatum* the pigment-dispersing effect preceded the pigment-concentrating one (lower portion of Figure 7). In contrast, when extracts of sinus glands from the dwarf crayfish, *Cambarellus shufeldti,* were assayed, concentration of dark red pigment occurred before dispersion (Fingerman, 1959). Red pigment in crayfishes becomes maximally dispersed after eyestalk removal (Brown, 1948). Crayfishes do not possess melanophores. Further investigation may reveal (1) the significance of this difference in behavior of the pigment-dispersing and pigment-concentrating principles in *Sesarma reticulatum* and *Cambarellus shufeldti* and (2) the reason why after eyestalk ablation the dark pigment concentrates in some crustaceans and disperses in others.

Summary and Conclusions

1. The grapsoid crab *Sesarma reticulatum* displayed a rhythm of color change which operated to disperse the pigment in the melanophores by day and concentrate it at night.

2. The crabs darkened as the intensity of illumination increased. A background response was also apparent. Melanin was more dispersed in specimens on a black background than on a white one, especially at low light intensities.

3. The degree of dispersion of the pigment in the melanophores of eyestalkless crabs was a direct function of the incident illumination. In dim light the pigment was maximally concentrated and in bright light was nearly maximally dispersed.

4. The melanin was less dispersed at high temperatures than at low. A thermoregulatory function of the melanophores was postulated.

5. Migration of melanin in *Sesarma reticulatum* is mediated by pigment-dispersing and pigment-concentrating principles.

6. The results were discussed in relation to pertinent data concerning the melanophores of crabs which have been investigated previously.

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THE CARBOHYDRATE COMPOSITION OF THE GLYCOGEN BODY OF THE CHICK EMBRYO AS REVEALED BY PAPER CHROMATOGRAPHY [1]

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The glycogen body is the term used to designate a unique mass of tissue found at the lumbo-sacral level of the avian nerve cord (Terni, 1924; Watterson, 1949). In the chick, this structure arises at 7–8 days of incubation, accumulates an appreciable amount of "glycogen" during the remainder of development and persists after hatching (Watterson, 1949; De Gennaro, 1959). The function of the glycogen body is unknown. Moreover, other questions about that structure remain unanswered at the present time. To date, there has not been any attempt to identify the carbohydrate composition of the glycogen body. It is assumed from the quantitative studies of Doyle and Watterson (1949) and Watterson *et al.* (1958) that the polysaccharide stored by that tissue is composed of glucose, on the basis of chemical tests which determine the reducing power of the hydrolysate. Such tests do not distinguish between glucose and other reducing sugars which might be present (Hawk *et al.*, 1954). It would seem important for any future work aimed at disclosing a possible metabolic function for the glycogen body to learn whether the polysaccharide stored by that structure is composed of glucose or other carbohydrates. It was felt that such information could be learned if glycogen was extracted from the glycogen body, hydrolyzed and the products of the hydrolysate separated by means of paper chromatography using known carbohydrates as controls.

Materials and Methods

Biological and chemical

White Leghorn eggs were incubated at 38° C. and in a relative humidity of 50–60%. The lumbo-sacral regions of the spinal column of 19-day chick embryos (stage 45 of Hamburger and Hamilton, 1951) were quickly removed and put into absolute alcohol at 4° C. The vertebral column of each piece was slit open to allow rapid fixation of the glycogen body. The glycogen body was then isolated from each nerve cord after 24 hours and transferred to a 20-ml. test tube. Thirty glycogen bodies were disintegrated in 5 ml. of 30% KOH in a boiling water bath for 30 minutes. After cooling, an equal volume of absolute alcohol was added to precipitate glycogen, and the mixture was left to stand at room temperature for two hours (Hawk *et al.*, 1954). The mixture was then centrifuged for 10 minutes at 2500 rpm and the supernatant was discarded. The sediment was washed

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twice with 70% alcohol, centrifuged as before and the supernatants were discarded each time. The sediment was evaporated to dryness in the incubator at 38° C. for 10 hours.

Exactly 20 mg. of glycogen so prepared were hydrolyzed in 1 ml. of 1 N H_2SO_4 as recommended by Block *et al.* (1958) for two hours at 100° C. The amount of reducing sugar at the end of one and two hours was determined with Benedict's solution in order to ascertain that hydrolysis was complete. The hydrolysate was cooled to room temperature and passed through burets packed with 10 ml. of ion exchange resins (Amberlite, IR-4B (OH) and IRC-50 (H), Fischer Co.) to remove salts and electrolytes which are known to induce artifacts in paper chromatography (Block *et al.*, 1958). Glycogen body samples were collected by repeated washings with distilled water and then concentrated to 5 ml. by evaporation under reduced barometric pressure. The amount of reducing sugar in the sample was determined with Benedict's solution. Aqueous solutions of d-glucose, galactose, fructose, mannose and ribose, which were used as standards for chromatography, were then made in concentrations equal to that of the glycogen body so that there would be a minimum of distortion in the size and color of spots on the paper chromatogram. Final concentrations of glycogen body and of the standards used in this study ranged between 0.3–0.5 g. per 100 ml.

Paper chromatography

One drop (0.02 ml.) of each standard and glycogen body were delivered with a micropipette at equally-spaced intervals along a base line 5 cm. from the short edge of paper sheets (Whatman no. 1, 20 × 40 cm. in size). Some carbohydrate standards were also mixed together and spotted on the same paper with glycogen body to serve as controls. Each sheet was dried and fashioned into a cylinder by stapling the long edges of the paper together. The base of each cylinder was immersed in a fingerbowl containing 50 ml. of solvent. The entire apparatus was covered with a battery jar which was sealed to a glass plate to insure a saturated atmosphere. The solvent used was that recommended by Glegg and Eidinger (1954) which consisted of the following mixture: 45 ml. n-butanol, 25 ml. pyridine and 40 ml. distilled water. Aniline oxalate and resorcinol-alcohol reagents were used to develop color (Block *et al.*, 1958). Chromatograms were sprayed with those reagents after they were removed from the solvent and dried for 20 minutes at 100° F. Some chromatograms were also sprayed with ninhydrin in order to learn whether amino sugars were present (Cramer, 1955).

Unidimensional, ascending chromatograms were run at room temperature for 24 hours and then studied for the separation and positions of spots occupied by the standards and glycogen body. The multiple development method, recommended by Block *et al.* (1958) and Glegg and Eidinger (1954) for separating mixtures of simple sugars and lower polysaccharides, was also used to learn whether further separation of the glycogen body was possible. In this method, ascending chromatograms were dried and returned to the solvent every 24 hours for a total of 96 hours.

Results

It was found that the position of each carbohydrate and of glycogen body was readily recognized on chromatograms after spraying with developing reagents.

Aniline oxalate stained all carbohydrates and glycogen body yellow except fructose, which gave no color, and ribose, which was pink. With resorcinol-alcohol, red spots were produced except glucose and glycogen body, which were not colored, and ribose which was stained brown. Spraying with ninhydrin gave negative results. The position of glycogen body corresponded only to that of the glucose standard in chromatograms developed after 24 and 96 hours in solvent. Effective separation of the mixtures of standards was attained after 96 hours of multiple development but did not occur with glycogen body (Fig. 1). Moreover, glucose

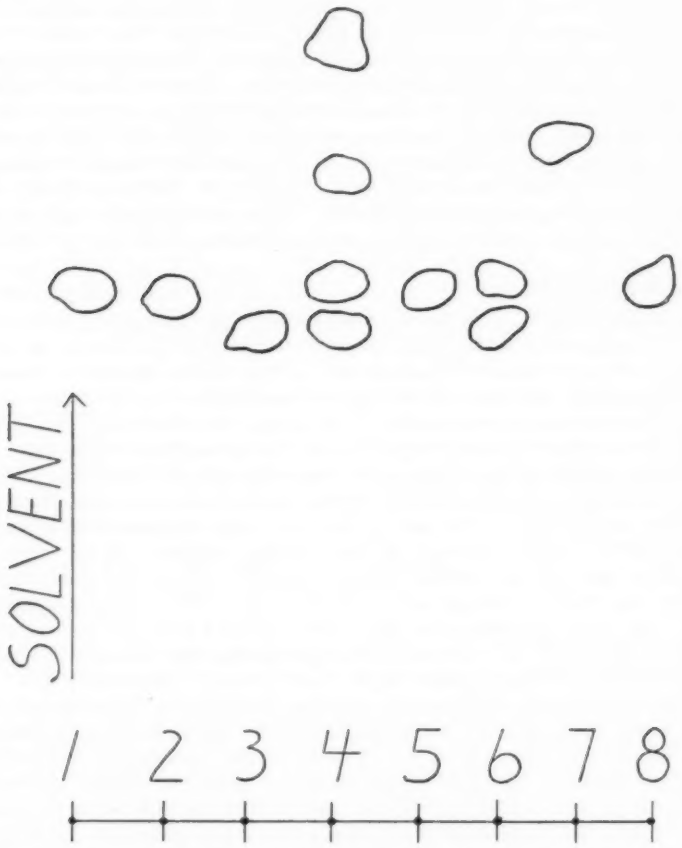

FIGURE 1. Chromatogram of glycogen body, carbohydrate standards and mixtures of standards obtained after multiple development of 96 hours. Effective separation of mixtures was attained after such treatment but did not occur with glycogen body. The position of the glycogen body corresponds only to that of the glucose standard. Explanation of numbers: 1, glycogen body; 2, glucose; 3, galactose; 4, galactose, glucose, fructose, ribose; 5, glycogen body; 6, galactose, glucose; 7, mannose; 8, glycogen body.

and galactose, which were often difficult to discern as individual spots on 24-hour chromatograms, are clearly separated from each other after 96 hours. These findings suggest that the polysaccharide stored by the glycogen body of 19-day chick embryos consists of glucose and not other carbohydrates.

Discussion

Doyle and Watterson (1949) have estimated that the glycogen body represents about 5–10% of the total glycogen of the chick embryo from 15 days of incubation to hatching. Attempts to prove that the glycogen body is a metabolic source of energy for the post-hatching period of the bird have not met with success in those experiments in which birds have been starved for various periods of time after hatching (Terni, 1924; Watterson *et al.*, 1958). Other experimentation designed to test whether the glycogen body supplies energy for morphogenesis or innervation of the posterior limb-buds has also yielded negative results (Jenkins, 1955; Watterson and Spiroff, 1949). Whether the glycogen body is metabolically inert is not known at the present time. The finding that the glycogen body consists of glucose and not other carbohydrates in this investigation might encourage a more positive view to suggest that the structure may have some importance. It would seem strange for a structure as large as the glycogen body to store vast quantities of glucose unless for some metabolic purpose. It is hoped that the results of the present study will stimulate research in that regard.

Summary

Glycogen was extracted from glycogen bodies of 19-day chick embryos with KOH and absolute alcohol, and hydrolyzed in H_2SO_4. The hydrolysate was analyzed for carbohydrates by means of paper chromatography, using known carbohydrates as controls. Special techniques are described which were employed in the preparation of the hydrolysate and for chromatography. The carbohydrate of the glycogen body was identified as glucose. Evidence is presented to suggest that the polysaccharide stored by the glycogen body of 19-day chick embryos consists of glucose and not other carbohydrates.

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CHANGES IN ABUNDANCE OF THE COMMENSAL CRABS OF CHAETOPTERUS

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The common marine annelid, *Chaetopterus variopedatus*, builds U-shaped, parchment-like tubes in the shallow waters along the Atlantic coast. The tubes are buried upright with only an inch or so of narrow siphon at either end showing above the surface of the substrate. Although the siphons are narrow, the tubes at their widest portion in the middle of the U may be as much as 4 cm. in diameter. Large tubes may reach a length of 85 cm. Of several commensal organisms that take advantage of the housing facilities set up by Chaetopterus, the most common and best known are two decapod crustaceans, *Polyonyx macrocheles* and *Pinnixa chaetopterana*. Seldom is a tube found without one or the other of these species being present. Usually adult crabs are in pairs, and especially is this true during the breeding season. A breeding pair of one species is rarely accompanied by an adult of the other species, although juveniles of the second species may often be present. The crabs are quite different; Pinnixa belongs to the family Pinnotheridae and Polyonyx to the family Porcellanidae. At the present time both species may be readily found in Chaetopterus tubes from Florida to southern New England, although this has not always been true for Polyonyx.

Enders (1905), while studying the development of Chaetopterus, noted the ratio of Polyonyx to Pinnixa at Beaufort, North Carolina. Pearse (1913) found a distinctly different ratio to exist in the region of Woods Hole, Massachusetts. These two authors appear to be the only ones who have made quantitative determinations of the relative abundance of Pinnixa and Polyonyx along the Atlantic coast. The present paper discusses the changes in relative abundance of these two crabs that have occurred in the last half century in the Beaufort and Woods Hole regions, reports their present abundance at Clearwater, Florida, and records observations on the life history of the crabs.

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Relative Abundance

Polyonyx has been called a southern crab, and it is true that its abundance on the American east coast was at one time far greater in southern than in northern waters. It was rare in the Woods Hole region at the beginning of the century.

One of the earliest records at Woods Hole is that of George M. Gray, late curator at the Marine Biological Laboratory, who found a specimen in a Chaetopterus tube in 1903. In marked contrast, about this same time, Enders (1905) found at Beaufort that approximately four-fifths of these two commensal crabs of Chaetopterus were Polyonyx. Sumner, Osburn and Cole (1911) mention Polyonyx as being present but apparently did not take any in their biological survey of the Woods Hole region. This is not surprising since Polyonyx, beyond the larval stage, is not known to occur outside Chaetopterus tubes and, with the dredging techniques used in the survey, few whole Chaetopterus tubes were obtained. However, Pearse, in 1913, found that Polyonyx constituted 22% of the crabs from 89 Chaetopterus tubes at Woods Hole. He comments on this apparent increase in abundance. Taylor, Bigelow and Graham (1957) used the increase in abundance

TABLE I

Relative abundance of Polyonyx macrocheles *and* Pinnixa chaetopterana *in three different regions*

Region	Tubes examined	Total crabs	Polyonyx		Pinnixa	
			No.	%	No.	%
Woods Hole, Mass.						
1913 (Pearse)	89	151	33	22	118	78
1959 (Gray)	120	205	136	66	69	34
Beaufort, N. C.						
1905 (Enders)	99	174	144	83	30	17
1958–59 (Gray)	383	740	289	39	451	61
Clearwater, Fla.						
1960 (Gray)	84	141	115	82	26	18

of Polyonyx in the Woods Hole region as an example of the spread of species due to amelioration of climate. These authors, who made no determinations themselves, quote Dr. John Rankin, then naturalist at the Marine Biological Laboratory, as saying that at the present time *Pinnixa chaetopterana* "is rare in the Woods Hole region, having been replaced by the southern form, *Polyonyx macrocheles*."

Questioning this statement of the scarcity of Pinnixa, an examination of 120 Chaetopterus tubes was made at Woods Hole in the summer of 1959. From the 205 crabs collected, a Polyonyx-Pinnixa ratio of 66:34 was obtained. This shows that while Pinnixa cannot be considered rare in the Woods Hole region, it has to a very considerable extent been replaced over the past half century by Polyonyx. The change in relative abundance may well be due, as suggested by Taylor *et al.*, to amelioration of climate. The Chaetopterus tubes at Woods Hole in 1959 were collected from a mud substrate in Hadley Harbor, some from the intertidal zone, but most from ten to twelve feet of water. The comparative abundance of Polyonyx and Pinnixa in 1913 and 1959 is shown in Table I.

The relative abundance of these crabs at Beaufort has also changed, but in a very different way. Whereas Enders in 1905 had established a Polyonyx-Pinnixa ratio of 83:17, in 1958-59, based on 740 crabs, the ratio was found to have shifted to 39:61. Interestingly, the ratio found by Enders at Beaufort in 1905 is essentially

the same as the Polyonyx-Pinnixa ratio on a sandy beach at Clearwater, Florida, in 1960 (Table I).

While amelioration of climate may be a satisfactory explanation for the increased abundance of Polyonyx in the Woods Hole region, it does not account for the increase in Pinnixa in the Beaufort area. Enders undoubtedly took his Chaetopterus from Bird Shoal, a large, continuous, sand flat covering several square miles. The western edge of this area is known as Bulkhead Shoal; the eastern portion as Guthrie Shoal. The 1958-59 populations were from three different areas: (1) a mud flat of the Newport River, a salt water estuary; (2) Bulkhead Shoal, a mixture of sand and mud; and (3) Guthrie Shoal, with a substrate of sand and shell. That the ratio of Polyonyx to Pinnixa differed in the three areas is shown in Table II. Although both species were found in tubes from all three substrates, it is evident that there is a definite correlation between the abundance of Pinnixa and the prevalence of mud. The increased abundance of Pinnixa on the more muddy

TABLE II

Correlation between abundance of Polyonyx and Pinnixa and type of substrate at Beaufort

Substrate	Tubes examined	Total crabs	Polyonyx		Pinnixa	
			No.	%	No.	%
Sand-shell (Guthrie Shoal)	139	261	141	54	120	46
Sand-mud (Bulkhead Shoal)	133	274	94	34	180	66
Mud (Newport River)	111	205	54	26	151	74

substrates suggests that, for this species, at least, the crabs enter the tubes as juveniles rather than as planktonic larvae. Pinnixa is a mud crab. Although more commonly found there than elsewhere, it is by no means limited to Chaetopterus tubes. It is frequently found in the mud tubes of the annelid, *Amphitrite ornata*, and occasionally in tubes of other dwellers of mud and sand flats. It is not uncommon on the shoals behind the Outer Banks, near Cape Hatteras, North Carolina, although the tubes of Chaetopterus are rare in this locality. On the other hand, Polyonyx has been reported from Chaetopterus tubes only.

LIFE-HISTORY AND ECOLOGY

Statements to the effect that neither of these crabs, when adult, can escape from the tubes which they entered as larvae or juveniles need qualification. While it may be true that adult crabs, especially Pinnixa, cannot escape through the openings of young Chaetopterus tubes with narrow chimneys, it is not true of crabs in older tubes with wide chimneys. In the field Pinnixa has been observed on several occasions leaving chimneys of wide diameter when the tubes were disturbed in the process of removal from the substrate. Under experimental conditions in the laboratory crabs enter and leave the tubes. If the diameter of the chimney is too narrow the crab bites a hole in the parchment at the base of the chimney and makes his entrance or exit at this point. This behavior, rare with Polyonyx, has been observed often with Pinnixa. Pearse (1913) noticed that Pinnixa placed in

aquaria with Chaetopterus were able to find their way into the tube, although he did not observe how this was accomplished. Probably under natural normal conditions neither species would have reason for leaving the tubes.

In September and October, when a new generation of Chaetopterus is developing and the tubes are new and being expanded rapidly, only small crabs are to be found in small tubes. It is assumed that the young crabs grow with the Chaetopterus tubes and are not replaced later by other crabs. It is not known if the crabs live more than one year. Judging by the length of the breeding season and the sizes of the crabs carrying eggs, it is thought that each species may breed more than once during the summer, at least in the more southern part of the range. In the Beaufort region the egg-carrying season for Pinnixa ends in early September, but Polyonyx continues to carry eggs in December. On November 30, 1959, 80% of the adult female Polyonyx taken had eggs, but a month later, December 29, none were with eggs. Enders (1905) reported that Polyonyx in the Beaufort

TABLE III

Carapace width of mature Polyonyx and Pinnixa from three different regions. The minimum among females is for egg-bearing individuals. Measurements are in millimeters.

	Polyonyx				Pinnixa			
	No.	Max.	Min.	Ave.	No.	Max.	Min.	Ave.
Woods Hole, Mass.								
♂	67	11.3	8.0	9.9	32	15.1	7.3	12.3
♀	70	14.4	10.1	12.8	35	17.4	9.4	13.4
Beaufort, N. C.								
♂	143	11.1	7.1	9.2	220	15.0	6.9	12.0
♀	141	16.0	10.1	13.0	221	17.5	8.4	13.5
Clearwater, Fla.								
♂	55	9.2	6.4	7.6	12	12.0	8.5	10.5
♀	60	12.9	8.4	10.5	14	14.0	8.9	11.8

area was breeding on October 25, the date of his last field observation. Egg-laying for both Pinnixa and Polyonyx in the Beaufort region is resumed in April. An examination of crabs from three different habitats on March 28-30, 1960, showed none with eggs, but several adult female Polyonyx appeared ready to molt. Presumably molting precedes egg-laying. On April 22 all females of both species were carrying eggs in early stages of development. It is possible that this is a late date for egg-laying, since March, 1960, was an abnormally cold month, with water temperatures in Beaufort Harbor dropping to freezing on several occasions. Female crabs of both species taken February 20 at Clearwater, Florida, were carrying eggs. When egg-laying begins or ends in the Woods Hole region was not determined.

In the summer months, while females are carrying eggs, there is usually one pair of either Pinnixa or Polyonyx, but not both species, present in any particular tube. If another species is present it is usually a small immature crab. However, beginning in mid-August in the Beaufort region, as new juvenile crabs appear, it is possible to find several young crabs, of either species, in the same tube with adults. In

August the majority of juveniles are Pinnixa. Forty-one of 49 juveniles from 100 Chaetopterus tubes, August 16-26, 1960, were Pinnixa. During the fall months the number of juveniles in Chaetopterus tubes, especially of Polyonyx, increases.

In both species females are larger than males. The carapace widths of crabs from Beaufort and Woods Hole are essentially the same (Table III). Crabs from Clearwater, Florida, averaged somewhat smaller. This is perhaps because the Florida specimens were taken in February, while the Beaufort and Woods Hole material was collected in July and August. In the Beaufort region it is thought that both Pinnixa and Polyonyx males are mature when they reach a carapace width of approximately 7 mm. The smallest females of Pinnixa with eggs measured 8.4 mm. and the smallest Polyonyx females with eggs 10.1 mm. However, Florida female Polyonyx as small as 8.4 mm. had eggs. It is not known if Pinnixa and Polyonyx taken in Florida in comparable numbers and in comparable seasons are in reality smaller than those from Beaufort and Woods Hole.

Living in protective Chaetopterus tubes the crabs are not subject to the same degree of predation or to the same fluctuations in environmental conditions that more exposed animals are. This is particularly true of those in the intertidal zone. Both Polyonyx and Pinnixa seek positions at the posterior end of the worm and are thus near one siphon or the other. In fall and winter months the siphons of Chaetopterus tubes are frequently cut off by the blue crab, *Callinectes sapidus*, and by birds, and undoubtedly many commensal crabs are then caught and eaten by predators. Temperatures within the tubes are more stable than on the sand flats. On a hot August day at Beaufort, when the temperature of water in small tide pools left at low tide registered 35° C., the maximum temperature within twelve Chaetopterus tubes tested was only 29°.

That Chaetopterus holds no strong chemical attraction for adult crabs has been demonstrated many times by students in the laboratory, but whether there is a chemical attraction for young crabs is not known. Experiments using the technique of Davenport (1950) in which a crab placed in a Y-tube has a choice of going upstream toward a jar containing Chaetopterus or toward a jar of sea water have produced negative results. Eighty per cent of the crabs failed to go in either direction. It seems probable that, at least for Pinnixa, the young crabs find their way by chance to the Chaetopterus tubes.

Of crabs other than Pinnixa and Polyonyx that invade Chaetopterus tubes in the Beaufort area, the mussel crab, *Pinnotheres maculata* is most often found. For the most part these are young males. The oyster crab, *P. ostreum*, occasionally with eggs, has been taken also. Rarely, the small spider crab, *Pelia mutica*, may be found. Usually these are in tubes bearing Pinnixa, seldom with Polyonyx. Pinnixa is not as clean a crab as Polyonyx. It commonly is covered with the bryozoan, *Triticella elongata*, and occasionally has young sea squirts, *Styela plicata*, attached to its legs or back. A hydroid, *Clava* sp., may also be found on Pinnixa. These sessile invaders are always attached to the crab, never to the wall of the Chaetopterus tube.

Discussion

It is difficult to offer a satisfactory explanation for the changes in relative abundance of the two commensal crabs, Pinnixa and Polyonyx. Amelioration of

climate, suggested by Taylor *et al.*, seems a satisfactory explanation for the change in distribution in the Woods Hole region. Here it is assumed that the shift in relative numbers has been a gradual one over a long period of time. However, the same explanation does not suffice for the shift in the opposite direction at Beaufort, where Polyonyx shows a marked decrease in relative numbers during the same period of time. At Beaufort two possibilities present themselves. There is no evidence one way or the other as to whether the change was gradual or sudden. If substrate is of importance, it is quite possible that Bird Shoal, where supposedly Enders secured his Chaetopterus, has changed materially in the past fifty years and perhaps has more mud mixed with sand at present than formerly. Considering the amount of dredging of the channels in recent years this seems entirely possible. In fact, dredging in the fall of 1959 added several inches of mud to the substrate on the inner side of Bird Shoal. The more muddy substrate could create an advantage for Pinnixa, which is quite at home in mud.

Another suggestion is that the change may have been sudden, following the great depletion of Chaetopterus as a result of a series of hurricanes in 1954 and 1955, which so extensively washed Chaetopterus tubes out of the substrate that they were scarce in the area in 1956 and only moderately plentiful in 1957. Chaetopterus did not again appear in abundance until 1958. The three hurricanes of 1955 between August 11 and September 15 were particularly disastrous to Chaetopterus tubes. Coming at a time when many Pinnixa had finished breeding and when the abundance of juveniles was far greater for Pinnixa than for Polyonyx, the former species would have been favored as the one to populate new Chaetopterus tubes that might be forming. Large Chaetopterus tubes were very scarce following these hurricanes. Pinnixa, in contrast to Polyonyx, can rapidly burrow into the substrate. In the laboratory Pinnixa has been observed to disappear into the substrate almost immediately, while Polyonyx remained on the surface even after several days. If separated from Chaetopterus tubes, Pinnixa would therefore have a much greater chance for survival than would Polyonyx, which is not known to occur in the adult stage outside of Chaetopterus tubes.

It is not clear when the crabs enter the tubes, whether as planktonic larvae, as juvenile crabs, or both. Since young Chaetopterus tubes have only young crabs in them it is assumed that the crabs grow with the tubes. In the case of Pinnixa it is thought that the tubes are entered by juveniles, since the crabs are able to live outside of Chaetopterus tubes.

It is obvious that Pinnixa, although it seems to prefer Chaetopterus tubes to other environments, is not an obligate commensal of Chaetopterus; nor are the bryozoans, hydroids and sea squirts that are so frequently attached to Pinnixa obligates of the latter. On the other hand, however, Polyonyx, beyond the planktonic larval stage found only in Chaetopterus tubes, must be considered an obligate commensal of Chaetopterus. Other than obtaining food brought in by the host, the chief benefit appears to be protection. Polyonyx does not burrow and is thus at a disadvantage when liberated among predators on a sandy substrate.

Summary

1. The relative abundance of two species of commensal crabs of Chaetopterus, *Pinnixa chaetopterana* and *Polyonyx macrocheles*, has been determined in the

regions of Clearwater, Florida; Beaufort, North Carolina; and Woods Hole, Massachusetts. The marked changes in relative abundance of the two crabs that have occurred during the last half century at Beaufort and Woods Hole are discussed.

2. Comparative observations on the life-history and ecology of the two crabs are recorded.

3. Pinnixa is considered a facultative commensal and Polyonyx an obligate commensal of Chaetopterus.

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INTERACTIONS BETWEEN ARBACIA SPERM AND S^{35}-LABELLED FERTILIZIN[1]

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The sperm of *Arbacia punctulata* and other echinoids agglutinate reversibly when mixed with fertilizin of the same species. Fertilizin is the sole large-molecular constituent so far detected in the jelly coat which surrounds the sea urchin egg, and consequently any reaction between this substance and sperm is of interest in studies of fertilization. Although there is general familiarity with the basic facts of the agglutination reaction, many aspects require clarification. For instance, the molecular events accompanying reversal are quite obscure. Attempts to understand these reactions have been advanced by the use of serological models (Lillie, 1914; Tyler, 1948, 1959). These models have led to the view that fertilizin is a large multivalent molecule which combines with two or more sperm per molecule to bring about agglutination. An antigen-antibody type union is thought to form between sites on fertilizin and complementary sites on the sperm surface associated with a protein termed antifertilizin. Reversal of agglutination occurs, according to this view, when fertilizin is altered to a univalent state, *i.e.*, the multivalent molecule is fragmented into components with no more than one reactive site on each part. Phenomena analogous to this explanation of sperm reversal are found in demonstrations of univalent antibodies in nature (Heidelberger, *et al.*, 1940), and in the experimental conversion of multivalent antibodies to a univalent form (Tyler, 1945; Porter, 1959). Multivalent fertilizin has been converted to a univalent form by various agents: ultraviolet and x-irradiation, proteolytic enzymes, hydrogen peroxide, and heat (Tyler, 1941; Metz, 1942, 1954).

The idea that sperm reversal results from conversion of fertilizin from a multivalent to a univalent form implies that the agent(s) of this conversion is sperm or some component(s) of sperm. This has led to questions regarding the mechanisms of such conversion and the molecular nature of the product. As an approach to these problems the present studies, employing S^{35}-labelled fertilizin (Tyler and Hathaway, 1958), were undertaken. The high sulphate content of most fertilizins (Vasseur, 1947, 1948; Tyler, 1959), and the possible role of such sulphate in fertilizin agglutination of sperm (Vasseur and Carlsen, 1948; Vasseur, 1952), suggested the experiments described in this paper. These show that sperm bind, and subsequently release, fertilizin S^{35}. This uptake and release of S^{35} label is associated with fertilizin agglutination of sperm and its reversal. These results, and

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other preliminary observations, have been published earlier in abstract form (Hathaway and Tyler, 1958; Hathaway, 1959).

Materials and Methods

Arbacia punctulata for all experiments were provided by the Marine Biological Laboratory Supply Department.

S^{35}-labelled fertilizin was produced by the procedure of Tyler and Hathaway (1958). This involved the injection of a sea water solution of $Na_2S^{35}O_4$ into females previously shed of gametes. Three injections of 50 microcuries each were administered on alternate days, during which time the animals were maintained in aerated sea water abundantly supplied with algal food. During the six days of injection treatment the animals apparently incorporated the inorganic S^{35} into newly produced eggs. At the end of this time the animals were left overnight in running sea water, and were then caused to shed their eggs by means of KCl injection. After repeated washings in cold sea water, the eggs were subjected to a pH of 3.5 to 4.0, which dissolved the fertilizin from them. Such fertilizin preparations, after dialysis, were found to be highly radioactive and to give the usual reversible agglutination reaction with sperm. Aliquots of a single fertilizin preparation were utilized in all of the experiments described below.

Sperm were collected by electrical stimulation of exposed gonads. Routine washing of sperm consisted of suspending about 3 ml. semen in 12 ml. cold sea water. After centrifugation, the sperm were re-suspended in sea water to a final volume of 5 times the volume of packed sperm. Radioactivity was measured in a windowless gas-flow counter. The "soft" beta emission of S^{35} is masked by sperm; therefore radioactive assays in these experiments were always made on supernatant fluids only.

Results

S^{35} uptake by sperm

Ten experiments were performed to test the capacity of sperm to remove radioactivity from solutions of S^{35}-fertilizin. In these, 1 ml. each of sperm suspension and labelled fertilizin were mixed at 0° C. The mixtures were centrifuged 30 seconds later and a 0.5-ml. sample of the supernatant (No. 1) was removed for S^{35} assay. The results of the ten experiments are given in Table I (Columns A, B, C, and D). The S^{35} activity of a 0.5-ml. sample of control fertilizin solution (1 ml. fertilizin plus 1 ml. sea water) is given in Column A. Column B gives the activity of corresponding supernatants of fertilizin-sperm mixtures (1 ml. fertilizin plus 1 ml. ca. 20% sperm) obtained by centrifuging immediately after mixing. Column C incorporates an adjustment (see footnote below Table I). Finally, as seen in Table I, Column D, the supernatant of such sperm-fertilizin mixtures contained an average of $28.0 \pm 1.1\%$ of the initial radioactivity. Evidently the sperm bound 72% of the S^{35} label initially present in the fertilizin solution. The 28% of unbound fertilizin could represent excess fertilizin or labelled non-fertilizin contaminant.

Release of bound S^{35} label from sperm

Since preliminary experiments (Hathaway and Tyler, 1958) had indicated a release of bound S^{35} from sperm, further tests were performed. These utilized the

material remaining from the above ten experiments. Sperm were re-suspended in remaining supernatant and left 45 minutes at room temperature. These suspensions were then centrifuged, and the supernatant (No. 2) was assayed for S^{35} (Table I, Columns E and F). As seen in Column G, these supernatants contained an average of $72.7 \pm 2.5\%$ of the S^{35} in the system. This striking increase in supernatant S^{35} concentration is evidently a result of the release of bound S^{35} from sperm.

TABLE I

S^{35} in supernatants of sperm and labelled fertilizin mixtures*

Experiment number	A	B	C	D	E	F	G
	Control unabsorbed fertilizin cpm/0.5 ml.	Supernatant #1 actual cpm/0.5 ml.	Supernatant #1 adjusted** cpm/0.5 ml.	% total counts recovered in supernatant	Supernatant #2 actual cpm/0.5 ml.	Supernatant #2 adjusted‡ cpm/0.5 ml.	% total counts recovered in supernatant
1	308	87	78	25	374	263	85
2	308	98	88	29	333	238	77
3	335	119	107	32	348	253	76
4	335	108	97	29	338	244	73
5	356	123	111	31	303	224	67
6	356	104	94	26	257	191	53
7	311	72	65	21	333	233	75
8	311	84	76	24	318	226	73
9	311	107	96	34	302	221	71
10	311	91	82	29	336	239	77
Average				28.0 ± 1.1			72.7 ± 2.5

* Counts per minute (cpm).

** Adjusted to equal 25% of supernatant by allowing for relative volume of packed sperm: actual count $\times$ 0.90 (sample was 0.5 ml. out of a total of 2.0 ml., of which 0.2 ml. was packed sperm).

‡ Adjusted to equal 25% of supernatant and to allow for relative volume of packed sperm (Actual count $\times \frac{1.3}{4} \div 0.5$, since sample was 0.5 ml. of total volume of 1.5 ml., of which 0.2 ml. was packed sperm), and for S^{35} removed in first sampling $\left(+ \frac{\text{Actual count first sample} \times 0.90}{4}\right)$.

Absorption of S^{35} by pretreated sperm

Earlier experiments using sperm agglutination as the test for fertilizin have established that sperm fail to absorb agglutinin following treatment with excess fertilizin. It seemed of interest, then, to determine if sperm behave similarly with regard to S^{35} label.

Accordingly, a test was performed to see if sperm can bind S^{35} during a second mixing with labelled fertilizin. The experiment had four steps: (A) Sperm and fertilizin were mixed and immediately centrifuged, and the supernatant was assayed for S^{35} (Table II, Column A). (B) Sperm were re-suspended in supernatant for 45 minutes, re-centrifuged, and the supernatant again assayed (Column B). (C) Remaining supernatant was removed. Sperm were re-suspended in a fresh labelled fertilizin solution, immediately re-centrifuged, and the supernatant was assayed (Column C). (D) Sperm were re-suspended in the remaining supernatant for 45

minutes, re-centrifuged, and the supernatant was again assayed (Column D). Results show that the sperm in steps A and B absorbed and released S^{35} as expected, but that in steps C and D, following the initial fertilizin treatment, the sperm bound practically no S^{35}. Control sperm (treated with sea water rather than fertilizin in steps A and B) absorbed all but 36% of the label in step C (Table II, Column C). These results show that if fertilizin is included in steps A and B it causes alterations in sperm that greatly reduce their capacity to bind S^{35} in step C.

TABLE II

Recovery of S^{35} in supernatants of sperm treated twice with labelled fertilizin

	A	B	C		D	
	Experimental	Experimental	Experimental	Control	Experimental	Control
Actual counts/min./0.5 ml. of supernatant	84	318	339	121	378	213
Adjusted counts/min./ 0.5 ml. of supernatant	76*	226**	305*	109*	322**	166**
% total counts‡ recovered in supernatant	24	73	98	36	103	53

Columns represent successive supernatants. A, B, C, D, as described in text. Data in columns A and B appear as experiment 8 in Table I.

Control sperm were treated with sea water instead of fertilizin in steps A and B.

* Adjusted to equal 25% of supernatant by allowing for relative volume of packed sperm: Actual count × 0.90 (sample was 0.5 ml. out of a total of 2.0 ml., of which 0.2 ml. was packed sperm).

** Adjusted to equal 25% of supernatant and to allow for relative volume of packed sperm (actual count $\times \frac{1.3}{4} \div 0.5$, since sample was 0.5 ml. of total volume of 1.5 ml., of which 0.2 ml. was packed sperm), and for S^{35} removed in first sampling $\left(+\frac{\text{Actual count first sample} \times 0.9}{4}\right)$.

‡ There were 311 counts/min./0.5 ml. of unabsorbed fertilizin solutions. This value is used as a baseline from which calculations were made of % total counts recovered in supernatants.

Control sperm in step D did not release as much S^{35} as was expected on the basis of the previous experiments (*cf.* Table I). It has been shown previously (Hathaway and Tyler, 1958) that excessive sperm washing impairs the capacity of the sperm for subsequent release of bound materials. Possibly the centrifuging and stirring of control sperm in steps A–C similarly explain the reduced release of S^{35} in step D. It is interesting in this connection that Rybak (1955) observed that sea urchin sperm washed and centrifuged two times in sea water fail to reverse after agglutination with fertilizin.

Failure of released S^{35} to bind to fresh sperm

Experiments outlined in a previous section show that a large proportion of S^{35} label initially bound to sperm is subsequently released. Consequently an experiment was performed to examine the extent to which previously bound and released S^{35} can be taken up by fresh sperm. (A) Sperm and S^{35} fertilizin were mixed, immediately centrifuged, and the supernatant was assayed (Table III, Column A).

(B) Sperm were re-suspended for 45 minutes, re-centrifuged, and the supernatant was again assayed (Column B). (C) The supernatant was decanted and mixed with fresh sperm, centrifuged immediately, and the supernatant was assayed (Column C). (D) Sperm were re-suspended for 45 minutes, re-centrifuged, and the supernatant was assayed again (Column D). The results show that S^{35} was absorbed and released by sperm in steps A and B, as expected. However, in step C the fresh sperm absorbed only 10% of the S^{35} from the previously absorbed solution. The 10% absorbed may represent excess fertilizin that was not absorbed in the initial treatment (Column A). In any event, the sperm failed to absorb 90% of the S^{35} label in this second absorption, whereas the sperm in the first absorption took up all but 24% of the S^{35} activity. These results are most readily explained

TABLE III

S^{35} in supernatants reacted twice with fresh sperm

	A	B	C	D
Actual counts/min./0.5 ml. supernatant	81	298	154	152
Adjusted counts/min./0.5 ml. supernatant	73*	212**	139*	134**
% total counts recovered in supernatant	24	70	90	88

Columns represent successive supernatants A, B, C, D, as described in text.

In steps A and B unabsorbed fertilizin solution had 304 cpm/0.5 ml. In steps C and D, the fertilizin solution before absorption had 152 cpm/0.5 ml.

* Adjusted to equal 25% of supernatant by allowing for relative volume of packed sperm: Actual count × 0.90 (sample was 0.5 ml. out of a total of 2.0 ml., of which 0.2 ml. was packed sperm).

** Adjusted to equal 25% of supernatant and to allow for relative volume of packed sperm (Actual count $\times \frac{1.3}{4} \div 0.5$, since sample was 0.5 ml. of total volume of 1.5 ml., of which 0.2 ml. was packed sperm), and for S^{35} removed in first sampling $\left(+ \frac{\text{Actual count first sample} \times 0.9}{4}\right)$.

by assuming that the S^{35} activity taken up and subsequently released in the first absorption is not re-absorbed by the second treatment with sperm. Column D shows that there is a little change in the amount absorbed by the second sperm treatment even after 45 minutes.

Non-diffusibility of S^{35}-labelled substances

Supernatants from experiments 1, 2, and 3 (Table I) were dialyzed to determine if the S^{35} material would pass through cellophane. No S^{35} diffused through the membranes in any of the tests. These results probably mean that the S^{35} in supernatants is associated with a high molecular weight substance. Alterations of fertilizin by whole sperm apparently do not involve a release of free sulphate.

Absorption of S^{35} label by heterologous sperm

Experiments were performed to see if sperm of other species can bind S^{35}-labelled *Arbacia* fertilizin. Sperm of *Chaetopterus variopedatus, Placopecten*

magellanicus, and *Spisula solidissima* were used. As in other experiments, sperm and fertilizin were mixed and centrifuged immediately, and the supernatant was assayed for S^{35} (Table IV, Columns B and C). *Arbacia* sperm absorbed 68% of the fertilizin as expected. *Placopecten* and *Spisula* sperm also absorbed substantial amounts of activity (97% and 51%, respectively). *Chaetopterus*, on the contrary, failed to absorb S^{35} activity from the *Arbacia* fertilizin during the 30-second exposure.

To test for release of bound *Arbacia* fertilizin the sperm were re-suspended in their supernatants and again centrifuged after a 45-minute interval. As seen in Table IV, Columns D and E, *Arbacia* sperm released appreciable amounts of S^{35}. *Spisula* and *Placopecten*, however, failed to release activity. Furthermore,

TABLE IV

S^{35} in supernatant of mixtures of Arbacia fertilizin and heterologous sperm

Fertilizin mixed with the sperm of:	A Unabsorbed fertilizin cpm/0.5 ml.	Supernatant No. 1		Supernatant No. 2	
		B Adjusted** cpm/0.5 ml.	C % total radio-activity remaining in solution	D Adjusted‡ cpm/0.5 ml.	E % total radio-activity remaining in solution
*Arbacia**	335	107	32	253	76
Spisu'a	335	166	49	64	19
P'acopecten	356	12	3	11	3
Chaetopterus	311	310	100	6	6

* These data appear as experiment 3 in Table I.

** Adjusted to equal 25% of supernatant by allowing for relative volume of packed sperm: actual count × 0.90 (sample was 0.5 ml. out of a total of 2.0 ml., of which 0.2 ml. was packed sperm).

‡ Adjusted to equal 25% of supernatant and to allow for relative volume of packed sperm (actual count $\times \frac{1.3}{4} \div 0.5$, since sample was 0.5 ml. of total volume of 1.5 ml., of which 0.2 ml. was packed sperm), and for S^{35} removed in first sampling $\left(+ \frac{\text{Actual count first sample} \times 0.90}{4}\right)$.

Chaetopterus sperm absorbed 94% of the S^{35} activity in this part of the experiment. Evidently *Chaetopterus* sperm absorbed fertilizin quite slowly or did so only after damage following a preliminary centrifugation (Compare Columns C and E, Table IV). The results show that the heterologous sperm irreversibly bind the S^{35}-labelled *Arbacia* fertilizin.

Whether the S^{35} label is bound to the sperm surface or to the subsurface material remains to be established. The results with *Chaetopterus* suggest that damage by centrifugation is required. Such damage may also be a factor in absorption of the S^{35} label by the other heterologous species. In any event, none of the heterologous sperm gave an agglutination reaction when mixed with *Arbacia* fertilizin.

DISCUSSION

The use of S^{35}-labelled fertilizin permits a recognition of two separable steps in the complex reaction between fertilizin and sperm. These steps are (1) an

immediate absorption of fertilizin to sperm, and (2) a subsequent release of a large fraction of the absorbed materials. The occurrence of these two successive reactions is consistent with the visually observed agglutination and reversal of sperm by fertilizin. The S^{35} data are consistent, also, with previous work.

Absorption of S^{35} fertilizin

Absorption of fertilizin by sperm has been indicated by several methods. Sperm decrease the agglutinating activity of fertilizin solutions (Lillie, 1913; Tyler, 1941; Metz, 1945). Since the agglutinating activity is a property of fertilizin, these experiments suggest fertilizin absorption by sperm. Work with *A. lixula* (Monroy *et al.*, 1954) shows that sperm remove the carbohydrate constituent, fucose, from fertilizin solutions. The present studies show that sperm absorb sulphur constituents from fertilizin solutions. There seems to be little doubt that an immediate absorption of fertilizin to sperm is a phenomenon of general occurrence in echinoids.

Release of S^{35} fertilizin

The results of this study show that after the immediate absorption of S^{35} fertilizin there is a subsequent release of a large fraction of the S^{35}. This result suggests an enzymatic degradation of the absorbed fertilizin, in which parts of fertilizin molecules are released to the medium. There have been many reports which infer the existence of a fertilizin-splitting enzyme on sperm. The viscosity of fertilizin solutions decreases when sperm or sperm extracts are added (Vasseur, 1951; Monroy and Tosi, 1952; Lundblad and Monroy, 1950). A later reevaluation of this work, however, led to the conclusion that viscosity decreases were due to absorption of fertilizin rather than to its enzymatic degradation (Monroy *et al.*, 1954). Other investigators have inferred the existence of fertilizin-splitting enzymes from examination of the effects of sperm extracts on the jelly coats of eggs (Hartmann *et al.*, 1940a, 1940b; Monroy and Ruffo, 1947; Numanoi, 1953). Although sperm extracts can be prepared which cause the disappearance of egg jellies, conclusions based on this observation must take into account the fact that sperm extracts can precipitate and contract the jellies rather than dissolve them. The necessity for distinguishing between such precipitating agents (antifertilizin) and other agents which might lyse the jelly coat and degrade fertilizin has been repeatedly stated (Tyler and O'Melveny, 1941; Krauss, 1950; Monroy and Tosi, 1952; Monroy *et al.*, 1954; Brookbank, 1958).

Experiments have been designed to distinguish between antifertilizin and suspected jelly-dispersing agents. Ishida (1954) observed that sperm treated with fertilizin still caused the disappearance of the egg jelly coat. Presumably the antifertilizin of these sperm was saturated with fertilizin so that the effects on the jelly coat were not due to antifertilizin. Brookbank (1958) has demonstrated a clear distinction between antifertilizin and an egg jelly-dispersing agent in *Mellita* sperm extracts. The jelly-dispersing agent is inactivated by acid (pH 4.0) and heat (70° C. for two minutes). Antifertilizin is not affected by heat or acid in the ranges used. The *Mellita* jelly-dispersing agent does not affect the sperm agglutinating titer of fertilizin in solution. The effects of this agent are limited to dispersing the jelly coat.

Recent experiments with *Arbacia punctulata* have indicated an egg jelly-dispersing agent from sperm. This agent is heat- and acid-labile (80° C. for 5 minutes, pH 3.0), and inactive at reduced temperatures (Hathaway, Warren and Flaks, 1960).

In the present experiments, whole sperm have been observed to divide fertilizin solutions into two fractions. One fraction appears to be bound permanently to sperm. The other fraction is only transiently associated with sperm. If the sperm are causing a fragmentation of fertilizin molecules, it might be expected that some parts of the molecule are freed from the sperm. The relative amounts of bound and freed S^{35} were roughly equal. The dialysis experiments support the idea that released S^{35} is associated with a large molecular type. Although these experiments do not conclusively show an enzymatic breakdown of fertilizin by sperm, the results are consistent with the hypothesis that an active fertilizin depolymerase is present in sperm.

A further possible explanation of S^{35} release by sperm is that some fertilizin molecules may be permanently adsorbed to sperm, while other fertilizin molecules combine with sperm receptors which themselves separate from the sperm. The complex of fertilizin and separated sperm receptors would then be recovered in supernatants. This fertilizin would have its reactive sites occupied by sperm receptors and would not react with additional sperm. The release of sperm receptors during reversal of agglutination was first suggested by Lillie (1914). It is known that sperm substances, presumably from the cell surface, are "soluble" enough to be detected in the medium (Monroy *et al.*, 1954; Köhler and Metz, 1960). It has also been demonstrated that fertilizin solutions cause the release of sialic acid-containing constituents from sperm (Warren, Hathaway and Flaks, 1960). Further experiments are required to decide if either of the suggested mechanisms is operative in the reaction of sperm and fertilizin.

Sperm agglutination and fertilization

The uptake and release of fertilizin S^{35} by sperm presents a partial explanation of the molecular events accompanying sperm agglutination and reversal. Agglutination evidently results from the binding of fertilizin to sperm. Reversal appears to be accompanied by the release of some S^{35}-containing fertilizin components. The release of S^{35} substance is evidence that reversal is caused by a breakdown of fertilizin.

There is an obvious value in a mechanism which would permit the egg to "trap" an approaching sperm. The immediate absorption of dissolved S^{35} fertilizin to sperm suggests that similar bonds hold the sperm trapped in the jelly coat of the egg. Once the sperm is trapped, however, it must proceed through the jelly coat to the egg membrane. Such a process would be facilitated by a sperm agent which would cause the breakdown of fertilizin. The agent which causes S^{35} release might serve this purpose in the case of fertilization in sea urchins.

The authors are grateful to Dr. Albert Tyler, California Institute of Technology, for much help and encouragement during the course of this work.

Summary

1. Reactions between *Arbacia* fertilizin and sperm were studied with preparations of S^{35}-labelled fertilizin. Experiments consisted of mixing fertilizin and sperm, centrifuging the sperm from the suspension, and assaying the supernatant for radioactivity. In this way the absorption of fertilizin to sperm could be measured as a loss of radioactivity from the supernatant.

2. Results of 10 experiments show that there was an initial absorption by sperm of all but 28% of the fertilizin. This was rapidly followed by a release of more than one-half the absorbed material.

3. Sperm which have reacted with fertilizin do not absorb additional fertilizin upon further treatment with this substance.

4. Fertilizin solutions which have reacted with sperm are not appreciably absorbed by fresh sperm.

5. Dialysis shows that the S^{35}-labelled material absorbed to and released from sperm is non-diffusible.

6. Heterologous sperm from three species absorb *Arbacia* fertilizin but do not release it.

7. The parallel between absorption and release of S^{35} by sperm, and the agglutination and reversal of sperm by fertilizin, is noted, and it is concluded that the behavior of S^{35}-labelled material in these experiments is a molecular expression of the reversible agglutination reaction.

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RESPIRATION RATES IN PLANARIANS. II. THE EFFECT OF GOITROGENS ON OXYGEN CONSUMPTION [1]

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A number of drugs, known as goitrogens, interfere with one or the other of the mechanisms whereby the thyroid hormones are synthesized. Whether this interference is accomplished by blocking iodination of tyrosine or by inhibiting the oxidative coupling of iodotyrosines to form iodothyronines has not been determined (Astwood, 1955).

The administration of goitrogens to mammals results in thyroid hyperplasia and a distinct decrease in basal metabolic rate (Astwood *et al.*, 1943), but few investigations have been made on the effect of these agents in invertebrates. No studies have been reported on the effect of these chemicals on metabolic rate in planarians as evidenced by oxygen consumption.

The present work is a study of the effect of goitrogens on oxygen consumption in *Dugesia dorotocephala*, a common fresh water planarian.

Design of Experiment

Planarians were collected from Buckhorn Springs [2] in Murray County, Oklahoma, and kept in the laboratory for several weeks before this series of experiments was begun. The worms were maintained in aerated lake water in deep, white-enameled pans, at a temperature of 20° C.

Three goitrogens were used in this study, as follows: 0.005 *M* and 0.0025 *M* thiourea, 0.0005 *M* and 0.00025 *M* phenylthiourea, and 0.005 *M* and 0.0025 *M* thiouracil. These concentrations were found not to be toxic to planarians regenerating in the solutions for a period of one week.

Worms not fed for seven days were subjected to the higher concentrations of each chemical; lower concentrations were used for animals not fed for eight days before the beginning of the experiment. The choice of the seventh and eighth day after feeding was made on the basis of earlier experiments (Jenkins, 1960) which demonstrated that phase *C*, the period of most gradual decline of metabolic activity in planarians which occurs some time after feeding, was established in these animals after about the sixth day.

On the day that an experiment was begun, thirty of the largest specimens were chosen from one stock pan and examined with a binocular microscope, then

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[2] Acknowledgment and thanks are due to Oscar Lowrance, owner of Buckhorn Springs property, for permission to collect the planarians.

divided randomly into six groups of five worms. Each group was thereafter treated as a unit. Six replicates were used for each series of experiments performed.

The first determination of oxygen consumption, on Day 0, was made with the six groups of animals in water. At the close of the readings the worms were weighed, then placed in labeled fingerbowls, each containing 100 ml. of the goitrogen solution to be used for that phase of the study. Readings on the second, fourth and sixth days were made with the animals in the same solution as that in which they had been living. On the sixth day, after readings had been made, three of the six groups of animals were returned to water, the other three being kept in the chemical. The final two measurements were made on the eighth and tenth days.

For determinations of oxygen consumption, the Warburg respirometer was used. After a thirty-minute equilibration period, readings were taken every half-hour for three hours. The respirometer was kept in a constant temperature room so that the experimental animals were subjected continuously to a temperature of 20° C. At the termination of the day's readings, each group of worms was weighed on a Roller-Smith torsion balance accurate to 0.2 mg. They were then returned to clean fingerbowls containing fresh solution.

Two series of oxygen consumption measurements on controls kept in water were made in a similar manner, one for worms starved seven days, and the other for those without food for eight days.

A second set of experiments was performed to test whether or not the goitrogens would cause an increase or decrease in the oxygen consumption of the planarians immediately after they were placed in the chemical. The same concentrations were used, and the procedure was similar to that outlined above, with the following exceptions: After the first measurement in water on Day 0, the worms were returned to water for 24 hours. For the second measurement, on Day 1, the worms were placed in reaction flasks prepared with the proper goitrogen, and measurements of oxygen consumption during the first 3½ hours in the chemical were made. The worms were then placed in the drug overnight, and the third and final readings taken after 24 hours' exposure.

Results

Data secured with these treatments were calculated according to standard methods (Umbreit *et al.*, 1949) and the results expressed in microliters of oxygen consumed per gram of wet weight per hour. The values obtained with the six replicates were subjected to a statistical analysis of variance and found to be significant at $P < .001$.

Both thiourea and thiouracil depressed oxygen consumption slightly but not significantly below the usual gradual decline found in planarians subjected to prolonged starvation (Jenkins, 1960). A return to normal levels was established within four to six days in the animals exposed to the goitrogens for a prolonged period.

Planarians placed in 5×10^{-4} *M* phenylthiourea showed a significant fall in oxygen consumption within the first 3½ hours after they were placed in the goitrogen (Table I). In the animals kept in phenylthiourea for a prolonged period, a return to normal levels occurred within two to four days, followed by a gradual

TABLE I

Effect of goitrogens on oxygen consumption of planarians within 27½ hours after initial exposure

Treatments	μl. O_2/gm./hr.		
	Days		
	0	1	2
Water	140	147	142
Thiourea, 5×10^{-3} *M*	121	122	135
Thiourea, 2.5×10^{-3} *M*	120	117	127
Phenylthiourea, 5×10^{-4} *M*	143	116	127
Phenylthiourea, 2.5×10^{-4} *M*	138	105	119
Thiouracil, 5×10^{-3} *M*	141	143	136
Thiouracil, 2.5×10^{-3} *M*	132	140	133

rise which was significantly above normal by the sixth day, the maximum being reached on the eighth day. The effect was similar in the lower concentration, but less pronounced. A return to water slightly diminished the rise in oxygen uptake, but a return to normal levels was not established within four days.

The effect of the higher concentrations of goitrogens on the respiratory rate of the planarians as compared with the usual gradual decline exhibited by the water controls is illustrated in Figure 1.

DISCUSSION

The mechanism of goitrogenic action has not been completely elucidated, but current evidence favors the view that these compounds, by virtue of their reducing activity, produce their effect in the vertebrate thyroid by interfering with the conversion of iodide to iodine, presumably by inhibiting a peroxidase (Astwood, 1955), although the presence of the latter in the thyroid has not been demonstrated.

It is possible that the action of the goitrogens in depressing oxygen consumption in planarians is mediated through a similar mechanism. Although the fall in

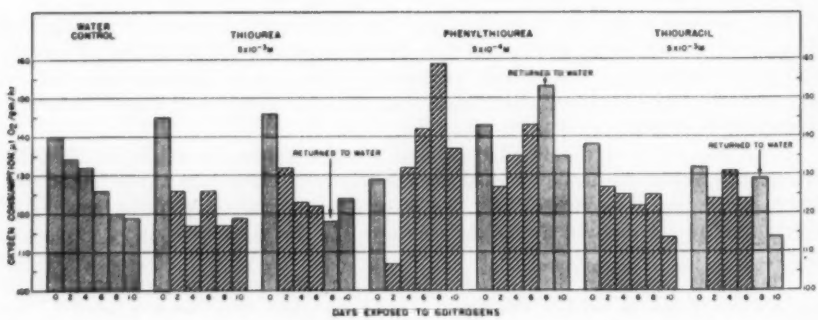

FIGURE 1. Effect of higher concentrations of goitrogens on oxygen consumption of planarians.

metabolic rate with thiourea and thiouracil was statistically insignificant, the depression in rate occasioned by the use of phenylthiourea was pronounced and continued for several hours. Jones and Wilson (1959) found the phenylthiourea-induced fall in the rate of oxygen consumption in *Cynthia* silkworm larvae and pupae was accompanied by an inhibition in the blood phenolase (tyrosinase) activity, and suggested the possibility of this enzyme being implicated in some way with respiration. Its action is blocked by a number of goitrogens, including thiourea, phenylthiourea and thiouracil.

Tyrosinase has not been demonstrated in planarians, but it is found in many invertebrates. Its presence in planarians is suggested, however, by reports of the effectiveness of thiourea compounds in causing eye depigmentation. Kambara (1954) found that pigment granules in eye-spots disappeared as a result of thiourea treatment, but skin pigment was not affected. His results were confirmed by Teshirogi and Maida (1956) and Kido and Kishida (1960). Jenkins (1959) noted that the formation of pigment was inhibited completely in the eyes of planarians allowed to regenerate in solutions of phenylthiourea at various concentrations. In the planarians returned to water, a steady development of pigment occurred. The colors appeared in the order commonly noted in melanin formation, namely tan, red to reddish-brown, and finally black. In view of these facts, it appears quite possible that the depressant action of phenylthiourea on oxygen uptake in planarians may be due to the inhibition of tyrosinase by the goitrogen.

The stimulatory effect of the phenylthiourea on planarian respiration, after prolonged exposure, is presumed to be due to the toxicity of the goitrogen. The reversal of the initial depression, which was followed by a pronounced increase in respiratory rate, was accompanied by some degree of cytolysis. Although an effort had been made to use only physiologically tolerated concentrations, in the 5×10^{-4} *M* phenylthiourea, on the tenth day after the worms had been put into the chemical, one worm had several small lesions and five others had one or two small lesions each. None of the worms in the lower concentration of the drug showed any evidence of similar injury; nevertheless, a similar but less pronounced rise in oxygen consumption shows that they were affected. The cause, under the circumstances, is presumed to be the same in both instances.

It might be of interest to add that a possible effect of goitrogens on sexual activity in planarians was noted. During the time the experimental animals were kept in the solutions, both copulation and cocoon deposition occurred. Six pairs of copulants were seen in the thiourea and five in thiouracil, but none was observed in either water or phenylthiourea. Ten cocoons were deposited by the thiourea-treated animals, and one by the water controls, but none was found in either of the other chemicals. Two of the ten cocoons were deposited in the Warburg reaction flasks while measurements were being made.

The ten cocoons were transferred to separate bowls of thiourea solution and kept for four weeks. By the fourth day they had begun to lose their original creamy whiteness, but complete darkening did not occur. No worms emerged from any one of the ten.

Summary and Conclusions

1. A study was made of the effect of the goitrogens thiourea, phenylthiourea and thiouracil on oxygen consumption in planarians.

2. Thiourea and thiouracil were found to have no statistically significant effect.

3. With phenylthiourea there was a marked initial depression, then a gradual return to normal levels followed by a significant rise. In the animals returned to water, oxygen consumption was not reduced to normal levels within four days.

4. It is suggested that the depressant action of the phenylthiourea may be ascribed to its ability to inhibit tyrosinase, while the stimulatory effect may well be due to the toxicity of the goitrogen.

5. A possible effect of thiourea upon sexual activity in planarians was noted.

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PHOTORECEPTOR CELL RESPONSE AND FLICKER FUSION FREQUENCY IN THE COMPOUND EYE OF THE FLY, LUCILIA SERICATA (MEIGEN)

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Compound eyes of insects function over a wide range of light intensity and speed of movement. At one extreme are diurnal species such as the honeybee, dragonfly and blowfly, which can orient visually to their surroundings while in rapid flight. At the other extreme are crickets and walking sticks, which are nocturnal and seldom or never fly. Autrum (1950) was the first to point out that the compound eyes of species at the two extremes differ in electrophysiological properties, specifically in properties of the electroretinogram (ERG). Autrum and co-workers (Autrum, 1950; Autrum and Stoecker, 1950; Autrum and Gallwitz, 1951) cited the following properties as characteristic of the honeybee, dragonfly and blowfly: (1) a diphasic ERG with positive on-effect and negative off-effect; (2) flicker fusion frequencies (FFF) ranging from about 180/sec. to over 300/sec.; (3) relatively low sensitivity to light; (4) high rate of dark adaptation. In contrast, Autrum (1950) cited these properties as characteristic of the cricket and walking stick: (1) a monophasic, cornea-negative ERG; (2) FFF lower than about 60/sec.; (3) relatively high sensitivity to light; (4) slow rate of dark adaptation. The two extremes were designated "fast" and "slow" eyes.

My own data are in good agreement with Autrum's on FFF, though not with respect to wave form of the ERG, sensitivity, and rate of dark adaptation (Ruck, 1958a, 1958b). Attention here is directed to the area of agreement, namely that FFF may be several times higher in certain diurnal insects than in certain nocturnal ones. The ultimate physiological problem in this area is to explain the molecular mechanisms underlying the differences in FFF, but first it is necessary to identify the anatomical structures in which the mechanisms operate. Autrum (1952, 1958) has proposed that two layers of cells—the photoreceptor cells and postsynaptic neurons of the *lamina ganglionaris*—are essential to the production of high FFF in the fast eye. According to this hypothesis, the electrical response of the receptor cells evokes an electrical response in the *lamina ganglionaris*, and the two electrical fields then interact in such a way that desensitizing changes associated with light adaptation are kept to a minimum. An eye in which such electrical interaction occurs is capable of responding to more flashes per unit time than an eye in which the interaction is absent. Autrum (1952, 1958) proposes that photoreceptor cell responses are fundamentally similar in fast and slow eyes, and that differences in FFF are produced by addition of the *lamina ganglionaris* response to the fast eye only.

If the proposed mechanism actually operates in the fast eye, removal of the *lamina ganglionaris* response should result in a decrease of the FFF to values

characteristic of the slow eye. Autrum and co-workers assert that this is the case. However, the methods which they have used to remove the response of the *lamina ganglionaris* are subject to the criticism that that response could not, by the nature of the experimental operations, be proven to be the only one effected. Thus, surgical removal of the *lamina ganglionaris* (Autrum and Gallwitz, 1951) necessitates transection of the axons of the photoreceptor cells where they enter the *lamina ganglionaris*. The postoperative ERG's in such an experiment must indeed be dominated by responses of the photoreceptor cells, but the assumption that these responses are unaltered by surgery is questionable. Lowering the P_{O_2} (Autrum and Hoffmann, 1960) and application of nicotine (Autrum and Hoffmann, 1957) involved simultaneous exposure of photoreceptor cells and cells of the *lamina ganglionaris*. The assumption that only responses of the latter were affected is again questionable.

This paper is concerned with a method of isolating the photoreceptor cell response in the fast compound eye of the fly, *Lucilia sericata*, without altering appreciably the response of the *lamina ganglionaris*. It is found that the electrical response of the whole optic ganglion, of which the *lamina ganglionaris* is a part, actually produces no detectable potential difference across the layer of photoreceptor cells. The photoreceptor cell potential develops in the presence of the optic ganglion potential and is unaffected by it. The isolated photoreceptor cell potential has a FFF higher than 200/sec.

The electrical interaction hypothesis (Autrum, 1952, 1958) specifies that the electrical field associated with the response of the *lamina ganglionaris* acts to prevent or arrest the development of a sustained electrical response of the photoreceptor cells. In view of present findings that hypothesis appears to be untenable. The data suggest the alternative hypothesis that the photoreceptor cells of the fast eye are inherently capable of responding to more flashes per unit time than photoreceptor cells in the slow eye.

Methods

Flies of the species, *Lucilia sericata*, were collected locally. Each fly was anaesthetized for about two minutes with CO_2 and fastened to a small platform with Tackiwax (Central Scientific Co.) as shown in Figure 1. A ring of wax was formed about the neck to immobilize the head. The platform was oriented so that the long axis of the body was vertical, the front of the head facing directly upward. The preparation was then positioned beneath a dissecting microscope. A small wax cup was built up about the cornea of the experimental eye. The wax was manipulated with the aid of a wire whose temperature was maintained electrically at the melting temperature of the wax. A group of anterior ommatidia was left exposed in the bottom of the cup, and within this area a piece of cornea about four facets wide and eight long was dissected free from the subcorneal tissues.

An indifferent electrode (E_3), consisting of an electrolytically tapered stainless steel wire, was inserted into the intact compound eye so that its tip lay just beneath the cornea. This electrode was supported by a mass of wax attached to the same platform which bore the fly. After this electrode was placed, the intact eye was covered with aluminum foil which shielded the eye from the stimulating light.

The fly and platform with E_3 in place were then mounted on a micromanipulator with the long axis of the fly still in the vertical position. The preparation was

moved into position beneath the optical stimulator. The light source was a Sylvania glow modulator tube mounted in a flashlight barrel behind two lenses. The light beam converged to a spot focus and was directed vertically downward onto the preparation. A glass micropipette electrode (E_2), filled with 3 *M* KCl, was fixed to a second micromanipulator and aligned vertically. The tip diameter of E_2 was less than one micron. The tip of E_2 was positioned in the focus of the stimulating

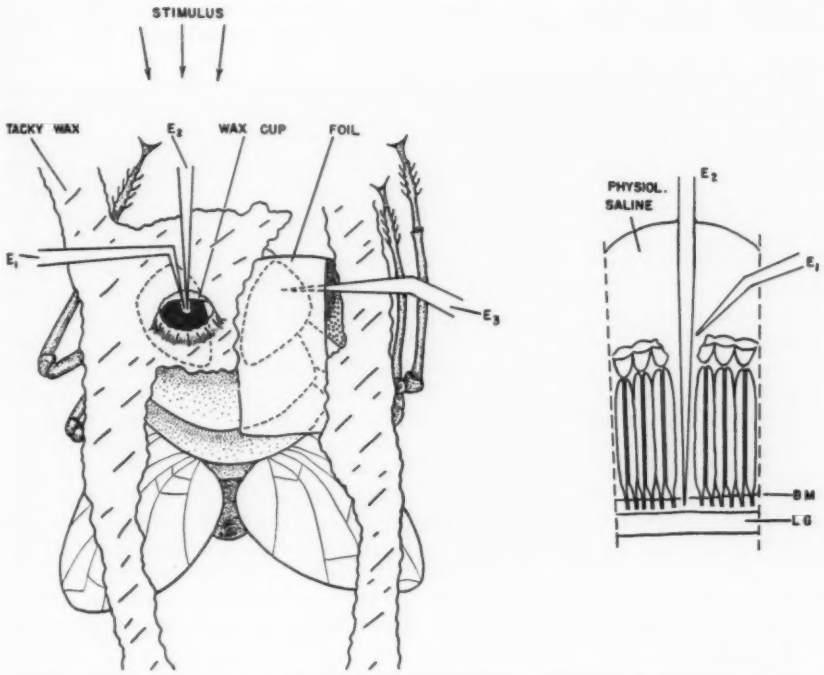

FIGURE 1. Left: *Lucilia sericata* in the experimental situation. Right: Diagrammatic section of the ommatidial layer to illustrate the positions of the electrodes in lead E_1–E_2. BM, basement membrane of the compound eye; LG, *lamina ganglionaris*.

light. The fly was then racked upward on its manipulator until the tip of E_2 just entered the area from which the cornea had been removed. The fly was not moved again during the experiment.

A third electrode (E_1), consisting of a tapered stainless steel wire coated to the tip with an insulating varnish, was moved into position next to E_2 with the aid of a third micromanipulator. A small drop of physiological saline was added to the wax cup. This maintained good electrical contact between E_1, E_2 and the preparation, and also prevented drying. At the beginning of an experiment the tips of E_1 and E_2 were located at the level of the cornea. E_1 remained at this position. E_2 could be advanced into the ommatidial layer.

The electrodes were connected to the input of a Grass P6 DC amplifier. Leads

E_1–E_2 and E_1–E_3 could be selected alternately with the aid of a switch. Responses were displayed together with a stimulus signal marker on the screen of a dual beam oscilloscope.

The glow modulator light source drew current from a DC power supply which was controlled by a Grass S4 physiological stimulator. At the beginning of an experiment the stimulus frequency was set at a constant one flash per second with a stimulus duration of 1/8 sec. Lead E_1–E_3—cornea of experimental eye vs. an electrically indifferent site—was selected first. Stimulus intensity was adjusted to produce a diphasic ERG with a positive on-effect and a negative off-effect. Then lead E_1–E_2 was selected. Since E_1 and E_2 were both at the level of the cornea at the start of an experiment, no responses could be recorded until E_2 was advanced into the ommatidial layer. As E_2 advanced, an ERG appeared and increased in amplitude with depth. This ERG was quite different in wave form from the one recorded in lead E_1–E_3. It consisted primarily of a cornea-negative wave. When this wave attained its maximum amplitude, a scale reading on the micromanipulator bearing E_2 was taken. A previous scale reading corresponded to the level of the cornea.

The path actually traversed by E_2 was determined at the end of each experiment. The fly was left in place and most of the dorsal ommatidia were stripped away from the head. This exposed the ommatidia along the path which E_2 had followed. This layer of ommatidia was viewed laterally with the aid of a dissecting microscope and the experimental penetration of E_2 was re-enacted by moving it along the same path and to the same depth as in the experiment. The position of E_2 at its deepest penetration in the experiments cited below was always at approximately the level of the basement membrane (Fig. 1). This position is an easy one to locate when the electrode is advanced blindly into the eye, because it corresponds to the maximum amplitude of the cornea-negative potential in lead E_1–E_2. Just beyond this position the ERG undergoes drastic alteration of wave form. At some depth within the layer of the optic ganglion corresponding to the *lamina ganglionaris* (Fig. 1) ERG's in lead E_1–E_2 become essentially like those recorded in lead E_1–E_3. The *lamina ganglionaris* is visible in the freshly dissected preparation as a white band of nervous tissue located close to the basement membrane of the ommatidial layer.

ERG's were recorded first across the ommatidial layer (E_1–E_2) under stimulus conditions which produce the diphasic wave form in lead E_1–E_3, then across the whole head (E_1–E_3) without changing the stimulus conditions, and finally across the ommatidial layer again (E_1–E_2) to determine whether the preparation remained in the same physiological condition. Using this sequence, responses to single flashes and to light flickering at 100/sec. and 200/sec. were recorded.

Stimulus intensities are given as equivalent footcandles illumination produced at the cornea of the experimental eye. Approximate values of illumination produced by the glow modulator were calibrated by a method described elsewhere (Ruck, 1961a). All experiments were conducted at room temperature which varied between 23° and 25° C.

Results

(1) Flies were mounted as shown in Figure 1. The stimulating light path was aligned with the visual axes of the anterior ommatidia. Preliminary responses

were observed using lead E_1–E_3. Stimulus duration was 1/8 sec., stimulus repetition rate one flash per second. The stimulus intensity was adjusted until the diphasic ERG illustrated in Figure 2,*b* was obtained. This is similar to the ERG Autrum (1950) describes as characteristic of the fast eye of the fly, *Calliphora*. Stimulus intensity was maintained constant at this level during the remainder of the experiment. Next, lead E_1–E_2 was selected. When both electrodes were at the level of the cornea, no response could be detected in this lead. E_2 was advanced until the ERG of Figure 2,*a* was recorded. The tip of E_2 then lay at about the level of the basement membrane of the ommatidial layer. The response across E_1–E_2 to a single 1/8-second flash (Fig. 2,*a*) consists of a sustained cornea-negative deflection

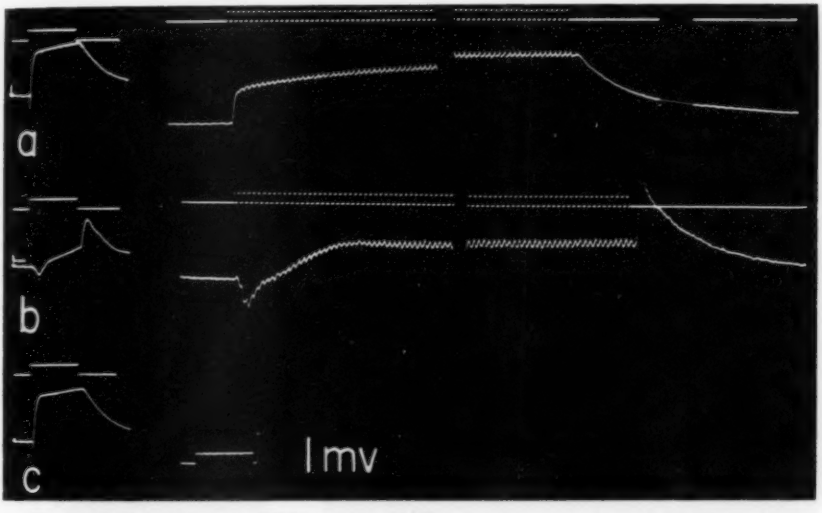

FIGURE 2. Comparison of ERG's in lead E_1–E_2 (records *a* and *c*) and lead E_1–E_3 (record *b*). For single flashes (left), stimulus duration is 1/8 second. Flicker frequency (right) is 100/sec. Stimulus intensity is constant (approximately 45 footcandles at the cornea) for all records. DC amplification.

plus a brief, cornea-positive on-deflection; this response is remarkably similar to responses obtained from the layer of photoreceptor cells in dorsal ocelli (Ruck, 1961a). A response to a light flickering at 100/sec. was recorded.

Next, lead E_1–E_3 was selected. In response to the 1/8-second flash the ERG is markedly diphasic (Fig. 2,*b*). It consists of a cornea-positive on-effect, a slight upward cornea-negative swing during illumination, and a cornea-negative off-effect. The response to a 100/sec. flicker stimulus is also shown.

Finally a control response was recorded, using lead E_1–E_2 (Fig. 2,*c*) in order to demonstrate that the condition of the preparation had not changed during the experiment.

The ERG's of Figure 3 were obtained from another animal. Only responses to light flickering at 200/sec. are shown. In lead E_1–E_2 (Fig. 3,*a*) the ERG is a monophasic, cornea-negative potential upon which are superimposed small waves,

one for each cycle of the stimulus. The recording obtained using lead E_1–E_3 (Fig. 3,*b*) also displays the responses to each cycle of the stimulus, but the wave form of the ERG is the diphasic type which Autrum (1950) described as characteristic of the fast eye. The recordings of Figures 3,*a* and 3,*b* were made at different amplifier gains so that the flicker potentials in Figure 3,*b* appear larger than those in 3,*a*. In order to compensate for this, a portion of each ERG was enlarged photographically (Figs. 3,*c* and 3,*d*) so as to make the vertical amplifications identical. At the same amplification the amplitudes of the flicker potentials are approximately equal. The experiment demonstrates that a flicker potential is as prominent in the absence of cornea-positive components of the ERG as in their presence.

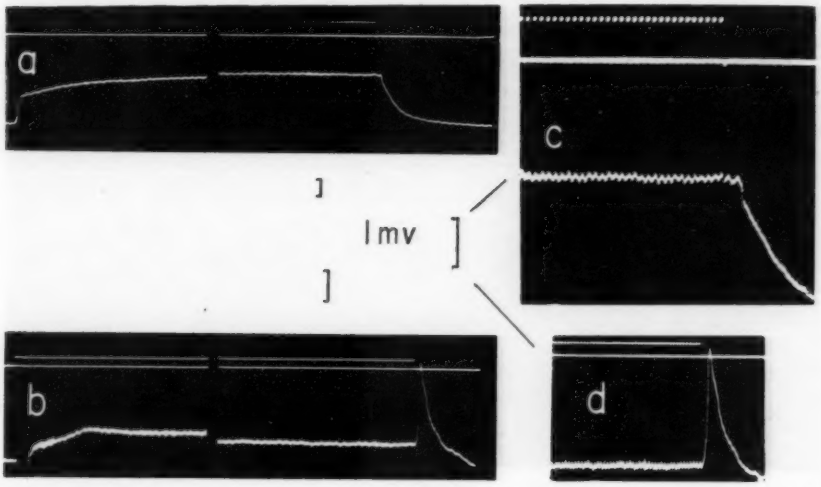

FIGURE 3. Responses in lead E_1–E_2 (records *a* and *c*) and in E_1–E_3 (records *b* and *d*) to light flickering at 200/sec. In *c* and *d* vertical amplifications of portions of responses in *a* and *b* have been made equal photographically. DC amplification.

Similar experiments were performed on 10 eyes. The only difference in results was that in five cases the brief, cornea-positive on-deflection in lead E_1–E_2 could be seen clearly (Fig. 2,*a*), and in the other five it could not (Fig. 3,*a*).

(2) All of the previous ERG's were recorded at the same stimulus intensity. The ERG's of Figure 4 illustrate the way in which wave form varies with stimulus intensity. All ERG's in Figure 4 were evoked by stimuli of 1/8-second duration, and all are steady-state responses to stimuli presented at a rate of 1/sec. Stimulus intensity increases from left to right in each row. In lead E_1–E_2 (Fig. 4, top row), the wave form is similar throughout the range of intensities. There is a sustained, cornea-negative potential plus a brief, cornea-positive on-deflection. The response simply increases in magnitude with stimulus intensity. Responses recorded with lead E_1–E_3 from the same eye immediately after the preceding series

are shown in Figure 4, middle row. The threshold response in E_1–E_3 is a monophasic, cornea-positive potential which does not appear at all in E_1–E_2. With increase in stimulus intensity it tends to overshoot the baseline as a negative off-effect. The brief, cornea-positive on-deflection seen in E_1–E_2 also appears in E_1–E_3. It is first visible in Figure 4,*b*, middle row. It has the same polarity in the two leads.

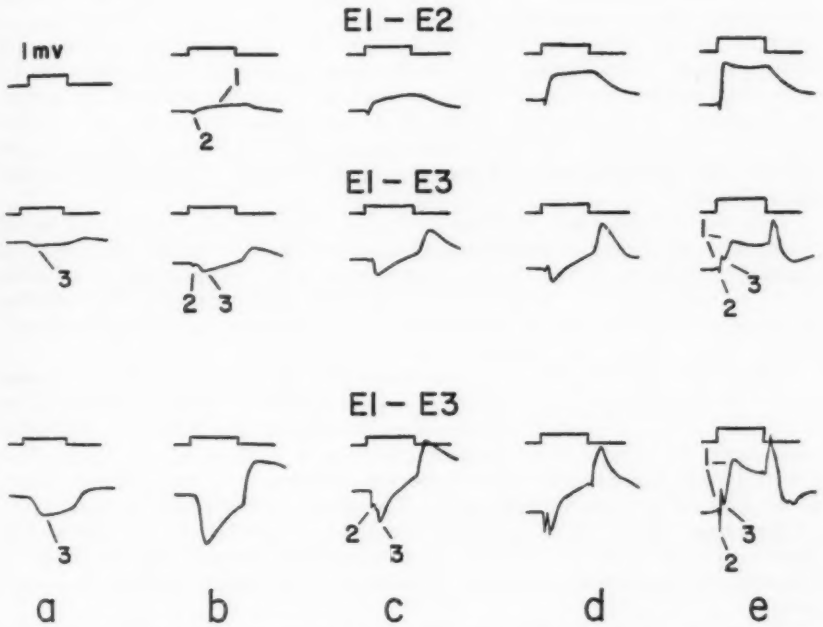

FIGURE 4. ERG's as a function of stimulus intensity. Leads indicated. Upper and middle rows from one eye, lower row from another. Stimulus duration, 1/8 second throughout. Stimulus intensity increases from *a* through *e*: *a*, 0.012; *b*, 0.12; *c*, 1.2; *d*, 46.1; *e*, 1200 foot-candles at the cornea. DC amplification.

Three components of the *Lucilia* ERG are numbered in Figure 4, using the same convention adopted in an analysis of the ocellar ERG of dragonflies and cockroaches (Ruck, 1961a). Components 1 and 2 appear in leads E_1–E_2 and E_1–E_3. Component 3 appears in E_1–E_3 only. The records of Figure 4, bottom row, illustrate a case in which components 2 and 3 were particularly conspicuous in lead E_1–E_3.

DISCUSSION

The cornea-negative component of the ERG (component 1, Fig. 4) appears in virtual isolation when only the ommatidial layer is included between the recording electrodes (E_1–E_2). One cornea-positive component (component 3, Fig. 4), which

is prominent when optic ganglion and ommatidial layer both lie between the recording electrodes (E_1–E_3), is excluded in lead E_1–E_2. These results are consistent with the interpretation that component 1 originates in the photoreceptor cells, component 3 in some deeper lying structure of the optic ganglion. The fact that component 3 cannot be recorded in lead E_1–E_2 indicates that the process responsible for that component produces no detectable potential difference between the cornea and the basement membrane of the ommatidial layer. Therefore, the only electrical interactions between components 1 and 3 are simple algebraic summations of potential wholly dependent upon the locations of the recording electrodes and without physiological significance. On the assumption that components 1 and 3 correspond respectively to the receptor cell and optic ganglion potentials of Autrum, his hypothesis (Autrum, 1952, 1958) of the mechanism of the fast eye response appears to be untenable. That hypothesis specifies that the electrical field associated with the optic ganglion potential prevents the development of a sustained receptor cell potential.

The negative off-effect recorded in lead E_1–E_3 (Fig. 4, middle and bottom rows) does not appear in lead E_1–E_2 (Fig. 4, top row). This off-effect, which in Autrum's hypothesis (1952, 1958) is regarded as a sudden release from inhibition of the receptor potential, actually originates in the optic ganglion. The off-effect is regarded here to be part of component 3. The receptor potential, component 1, simply decays toward the baseline at "off."

Component 2 is thought to originate in the photoreceptor cells. It was recorded in five out of ten preparations, in each of which it appeared together with component 1 in lead E_1–E_2. Component 3 was never recorded in this lead. A very similar component, also designated component 2, was found in dorsal ocelli (Ruck, 1961a, 1961b, 1961c). Component 2 was interpreted tentatively as a depolarizing response of the axons of the photoreceptor cells. The present interpretation of component 2 of the compound eye of *Lucilia* is the same, and depends upon the ocellar data. That is, the interpretation is based upon comparative evidence and the concept of homology of structure and function of retinula cells in dorsal ocelli and compound eyes.

Extending the comparative argument, component 3 may be interpreted as a hyperpolarizing, postsynaptic potential which arises at the junctions between retinula cell axons and second order nerve fibers of the *lamina ganglionaris*. The negative off-effect, if homologous with the similar component of the ocellar ERG, is a depolarizing after-potential—a sign of postinhibitory excitation—of the second order neurons in which component 3 originates.

A physiological mechanism which satisfactorily explains the difference in FFF between fast and slow eyes will probably be concerned mainly with properties of the retinula cells. The time would appear to be ripe for comparative studies of the responses and ultrastructure of individual retinula cells. Naka (1961) has begun work of this type and has obtained intracellular recordings from retinula cells of *Lucilia* which are consistent with interpretations of components 1 and 2 presented here.

The author would like to thank his wife for preparing the figures in this paper. The work was supported by a grant to Dr. K. D. Roeder from the United States

Public Health Service. Some of the equipment used was obtained under a previous contract between the Army Chemical Corps and Tufts University.

Summary

1. That part of the ERG which orginates in the retinula cells of the compound eye in *Lucilia sericata* was isolated without interfering with components originating in the optic ganglion. The retinula cell response is predominantly a sustained, cornea-negative wave, but in half the cases studied a brief, cornea-positive wave at "on" was also present.

2. Electrical events which originate in the optic ganglion produce negligible potential difference between the cornea and the basement membrane of the ommatidial layer. Therefore, an electrical effect, *per se*, of an optic ganglion process on the retinula cells is very improbable.

3. The electrical response of the retinula cells, uninfluenced by electrical fields produced by the optic ganglion, can resolve flicker stimuli of 200/sec. or higher.

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A STUDY OF THE EFFECTS OF URETHAN ON THE CLEAVAGE OF THE CHAETOPTERUS EGG. I. INHIBITION OF CLEAVAGE[1]

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Urethan (ethyl carbamate) is known to act as a potent antimitotic agent against cells derived from a wide variety of plant and animal tissues (Cornman, 1954). In addition, as a result of the pioneer work of Haddow and Sexton (1946), urethan has been used extensively in the chemotherapy of clinical and experimental tumors (Greenstein, 1954; Homburger and Fishman, 1953).

However, several lines of investigation have to date failed to produce conclusive information concerning the mechanism by which urethan inhibits cell division. Warburg (1956) suggested that urethan prevents cell division and arrests tumor growth of blocking cellular respiration. The theoretical basis of this hypothesis has generated a great deal of spirited debate in the literature (Weinhouse, 1955; Weinhouse *et al.*, 1956), but has not yet been proved experimentally. In addition there is some evidence that urethan may inhibit a biosynthetic pathway that is essential for cell division. In microorganisms and in tissue culture the administration of urethan or other carcinostatic substances results in the inhibition of cell division and the production of giant cells (Lasnitski, 1949; Sato, Belkin and Essner, 1956; Sirtori, Parvis and Pizzetti, 1955). Recent work has suggested that urethan may inhibit the biosynthesis of pyrimidines (Bresnick, 1960) and nucleic acids (Skipper, 1950; Skipper *et al.*, 1951). More experimental work is necessary, however, before the significance of these observations can be evaluated properly.

The present study was undertaken to investigate the possibility that urethan may be acting directly upon the physiological mechanisms responsible for the assembly of the mitotic apparatus. The eggs of marine invertebrates constitute an excellent experimental system for this type of work. Large numbers of cells can be readily obtained, which undergo division with almost perfect synchrony following fertilization. Experiments with very young embryos are presumably not complicated by problems of *de novo* net synthesis of proteins and nucleic acids (Brachet, 1957) or by problems of growth (Swann, 1957). However, these cells do convert storage proteins and nucleic acids into protoplasmic components, and this process may introduce complications.

Heilbrunn (1956) and his students have intensively studied the physical proper-

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ties of the cytoplasm of marine eggs during the mitotic cycle. A sharp increase in relative cytoplasmic viscosity, the mitotic gelation, takes place just prior to the cytological appearance of the mitotic apparatus (Heilbrunn and Wilson, 1948). Mazia (1958a) has shown that the mitotic apparatus is formed by the mobilization of soluble phase macromolecules that are already present in the unfertilized egg. Eggs treated with a wide variety of carcinostatic substances do not divide, and the mitotic gelation preceding the formation of the mitotic apparatus does not take place (Heilbrunn and Wilson, 1957; Heilbrunn *et al.*, 1957). The mitotic gelation may, therefore, reflect a generalized aggregation of macromolecules out of which the ordered structure of the mitotic apparatus is ultimately formed.

The experiments to be described indicate that in marine eggs urethan acts directly upon the mechanical structures of the dividing egg, the mitotic apparatus and the cortex. These experiments have been reported briefly elsewhere (Schuel, 1957; Schuel, 1958; Schuel, 1959).

I am indebted to the late Dr. L. V. Heilbrunn for suggesting this problem to me, and for his continual help and encouragement during the course of this investigation. I also want to thank Dr. Lester Goldstein for his guidance in the preparation of the manuscript.

Dedicated to Dr. L. V. Heilbrunn, my teacher and my friend.

Materials and Methods

The eggs of the marine annelid *Chaetopterus pergamentaceus*, which are available at Woods Hole, Mass., during the summer months, were used.

Gametes can be readily obtained from the worms by cutting the parapodia (for details, see Costello *et al.*, 1957). The eggs were filtered through several layers of cheesecloth and washed at least three times with sea water before being used. The eggs were maintained at 21° C. in a constant-temperature bath for the duration of the experiments. At this temperature about 50% of the eggs have divided at 56 minutes following insemination. In order to determine the percentage of divided cells, samples of 50 eggs were counted. This procedure made it possible to make rapid measurements of events taking place in populations of eggs maintained under a variety of experimental conditions, and the results from one experiment to another always indicated a definite and consistent trend.

Estimates of relative cytoplasmic viscosity were made according to the method described by Heilbrunn and Wilson (1948). A force of approximately 2250 times gravity was applied to the eggs by means of the hand centrifuge, and "relative viscosity" was judged by the number of seconds of exposure to this force required to produce a definite shift of light and heavy granules to opposite poles of the cell. During much of the time between insemination and first cleavage, 7 seconds are required to produce the visual impression of zones in the *Chaetopterus* egg with a force of this magnitude. About 27 minutes following insemination the cytoplasmic viscosity begins to increase, so that at 30 minutes about 14 seconds are needed for zone formation. The relative cytoplasmic viscosity remains high until the mitotic apparatus appears about 10 minutes later. Viscosity measurements must be made during this brief period in order to observe if urethan affects the

mitotic gelation. After the appearance of the mitotic apparatus, the viscosity returns to the basal level and rises sharply once again just prior to and during cytokinesis.

In order to visualize the mitotic apparatus within these eggs, cytological preparations were made with the help of Miss Jeanette Rose and Miss Jane Lehv. The eggs were incubated to the desired stage of the cleavage cycle, fixed in Bouin's fluid, dehydrated, sectioned (5 to 7 μ thick), stained with Heidenhain's or Harris' hematoxylin and mounted on permanent slides.

Results

I. Inhibition of division and the mitotic gelation

Chaetopterus eggs were put into solutions of urethan (ethyl carbamate) 5 minutes after insemination, and the results of this treatment on cell division and

Table I

The effect of urethan on cleavage and the mitotic gelation

Experiment	% Concentrated urethan	Relative viscosity at 30 minutes	% cleavage at 60 minutes
1	0.00	more than 14	98
	1.00	less than 10	0
	2.00	less than 10	0
	3.00	less than 10	0
2	0.00	more than 14	100
	1.00	less than 10	0
	2.00	less than 10	0
	3.00	less than 10	0
3	0.00	more than 14	92
	0.25	more than 14	94
	1.00	less than 8	0

The exposure to urethan was begun at 5 minutes after insemination and continued for the duration of the experiment.

Relative viscosity indicated by the number of seconds of exposure to a force of 2250 times gravity required to produce a shift of light and heavy granules to opposite poles of the cell.

relative cytoplasmic viscosity were observed (Table I). Preliminary experiments showed that urethan had to be present in concentrations greater than 0.5% in order to inhibit cell division. In supra-threshold concentrations of urethan the increase in relative cytoplasmic viscosity at 30 minutes, the mitotic gelation, was completely inhibited. The cytoplasmic viscosity of these eggs failed to rise above the basal level of 8 units and perhaps was even reduced. In 0.25% urethan, a sub-threshold concentration, the mitotic gelation took place normally and cleavage was not affected. There thus appears to be a correlation between the prevention of the mitotic gelation and inhibition of cytokinesis in this system.

II. Recovery of eggs on return to sea water

The urethan inhibition was reversible, at least in some cases. Eggs were put into solutions of urethan 5 minutes after insemination. At the end of an hour, when

all the control eggs had already divided, the inhibited eggs were returned to normal sea water and washed at least three times. The percentage of cleaved eggs was determined 15 minutes later. During this period relative cytoplasmic viscosity was estimated by means of the hand centrifuge. The results of typical experiments can be seen in Table II. At the end of 15 minutes almost all those eggs that had been in 1% urethan had divided, while only a portion of the eggs removed from 2% urethan recovered. Apparently the system is poised and ready to form the mitotic apparatus and complete division in the presence of urethan, and does so as soon as the inhibitor is washed away (see section IV below). Under these experimental conditions the inhibition by 3% urethan could not be reversed in any of the eggs. Upon return to sea water the 1% and 2% urethan-treated eggs divided into two blastomeres with the characteristic polar lobe, but the cells appeared to be somewhat "ragged" in appearance. The recovered eggs continued to divide and developed into very abnormal swimming larvae.

TABLE II

Recovery of inhibited eggs on return to normal sea water

Experiment	% urethan	Relative viscosity after 10 minutes	% cleavage after 15 minutes
1	1.0	more than 18	94
	2.0	more than 14	16
	3.0	less than 10	0
2	1.0	more than 16	88
	2.0	more than 20	16
	3.0	less than 10	0

Eggs were exposed to urethan 5 minutes after insemination and returned to normal sea water 55 minutes later.

Relative viscosity indicated by the number of seconds of exposure to a force of 2250 times gravity required to produce a shift of light and heavy granules to opposite poles of the cell.

Relative cytoplasmic viscosity determinations revealed somewhat surprising results. During the recovery period one might have expected the eggs to undergo cyclic viscosity alterations similar to those taking place normally during the mitotic cycle, a pre-mitotic gelation followed by a pre-cytokinetic solation and another sharp gelation just prior to and during cytokinesis. Several minutes following return to sea water the viscosity began to increase rapidly and sharply, culminating in cytokinesis within approximately 15 minutes. The maximum levels reached were considerably higher than those normally encountered during the mitotic gelation, but an accurate evaluation of the limits of this increase and its time course was not made because the cells were changing too rapidly. The pre-cytokinetic solation was thus absent, and the sharp increase in viscosity resembles closely the changes that have been reported by Heilbrunn and Wilson (1948) in the *Chaetopterus* egg during cytokinesis.

When the eggs were kept in urethan for longer periods of time, the first division following return to normal sea water was sometimes multipolar. In order to study this phenomenon quantitatively, eggs were put into urethan at 5 minutes after insemination and were returned to sea water at 30, 60, 90 and 120 minutes after

fertilization. The eggs returned to sea water at 30 minutes showed a cleavage delay of perhaps 5 minutes, and divided into two blastomeres. The eggs that were exposed to urethan for longer periods of time divided in about 15 to 20 minutes following return to sea water. At 60 minutes after insemination the controls were in the two-cell stage, and the eggs removed from urethan at this time divided into two blastomeres. By 90 minutes the controls had reached the four-cell stage, and the eggs removed from urethan at this time primarily divided directly into four blastomeres. Finally, the controls were in the eight-cell stage by 120 minutes, and the eggs removed from urethan at this time primarily divided into eight blastomeres. It thus appears that the eggs removed from urethan will divide directly into the number of cells that would have been produced by that time had there been no inhibitor present.

TABLE III

Exposure of eggs to urethan at various times during the cleavage cycle

Experiment	1% urethan added at minutes after insemination	% eggs going through	
		First cleavage	Second cleavage
1	No addition	97	91
	10	0	0
	20	0	0
	30	0	0
	40	0	0
	50	5	0
	60	98	6
2	No addition	94	88
	10	0	0
	20	0	0
	30	0	0
	40	0	0
	50	6	0
	60	88	2

III. Exposure of eggs to urethan at various stages of the cleavage cycle

The previous experiments have demonstrated that urethan inhibits the mitotic gelation and prevents cleavage. Was the mitotic gelation then the critical step in the division cycle for urethan inhibition? In order to study this question eggs were put into 1% urethan at 10-minute intervals during the first hour following insemination. It is clear (Table III) that urethan prevented eggs from dividing even after they had completed the mitotic gelation. All the eggs in the controls divided within 58 minutes after insemination. Second cleavage was prevented in those eggs that had completed first cleavage when they were exposed to 1% urethan (beginning at 60 minutes after fertilization). The small number of second cleavages observed in these experiments probably represented eggs that had divided directly into four blastomeres during the first cleavage cycle. A small percentage of multipolar cleavages may sometimes be observed in the controls.

It appears that there is no critical point in the mitotic cycle of these eggs where urethan can no longer block that division but inhibits the next. The existence of such a critical point has been reported by Mazia (1958b) with mercaptoethanol, and by other workers with diverse chemical and physical agents (for references see Swann, 1957).

However, it remained to be seen whether division could be completed by eggs which had begun to form cleavage furrows at the time they were exposed to urethan. In order to answer this question, fertilized eggs were incubated in sea water until about half of the population exhibited cleavage furrows, and were then exposed to a 1% solution of urethan. Table IV summarizes the results of these experiments. At first the furrowing process appeared to continue, since two minutes later all the eggs seemed to have formed two quite distinct blastomeres and the characteristic

TABLE IV

Reversal of cleavage furrows by urethan

% urethan	% cleavage furrows 60 min. after fertilization	% cleavage furrows 15 min. later
0.0	100	100
1.0	100	40
0.0	90	82
1.0	86	9
0.0	98	100
1.0	96	14
0.0	98	98
1.0	92	40

Eggs were exposed to 1% urethan when about half the population exhibited cleavage furrows (approximately 56 minutes after insemination).

polar lobe. However, the furrows soon began to regress and the cells reassumed a spherical shape, so that 15 minutes later only a small proportion of the eggs had completed cleavage planes. These few eggs probably had already completed cytokinesis at the time they were treated with urethan.

IV. Effect on the morphology of the mitotic apparatus

These results raise the problem of the fate of the organized structure of the mitotic apparatus in the presence of urethan. Normally the mitotic apparatus (centrioles and astral rays) is first visible in the *Chaetopterus* egg about 40 minutes after fertilization at 21° C. (Heilbrunn and Wilson, 1948). The experiments described above (section I) showed that eggs put into urethan at 5 minutes after insemination did not undergo the mitotic gelation and did not divide. One would predict that the mitotic apparatus was not formed.

This expectation was confirmed by the following experiment. Eggs were put into 1% urethan 5 minutes after insemination and fixed 40 minutes later. Control eggs fixed at the same time (45 minutes after insemination) appeared to be primarily in late prophase. The centrioles and the astral rays were sharply delineated at the poles of the cell while the nuclear membrane was in the process of breaking down. A few of the cells were in early metaphase. In the urethan-treated eggs no trace of the mitotic apparatus could be seen. In addition, a considerable number

of these eggs still contained male and female pronuclei. Urethan at this concentration apparently retards but does not prevent the fusion of the pronuclei. Similar results have been obtained with other narcotics (Painter, 1918; Wilson, 1901).

It has also been shown (section III) that fertilized eggs did not divide when put into urethan after the mitotic gelation had taken place or after the mitotic apparatus had formed. In this case would the organized structure of the mitotic apparatus be destroyed or would it prove to be morphologically intact but functionally inactive? To resolve this question, eggs were put into 1% urethan 45 minutes after insemination, and were fixed 5 minutes later. Control eggs fixed at the same time were in metaphase and early anaphase. In the urethan-treated eggs none of the structural components of the mitotic apparatus could be seen. A small oval-shaped area, apparently free of granules or other structures, was observed near the center of the eggs, and this probably represented the location of the destroyed spindle. In addition, no intact nuclei were observed, suggesting that urethan failed to inhibit the physiological mechanisms responsible for the dissolution of the nuclear membrane.

Fertilized eggs that had been treated with 1% urethan divided in about 15 minutes after return to sea water (section II, above). During the recovery period there was a sharp and rapid increase in relative cytoplasmic viscosity (Table II), similar to the pattern observed by Heilbrunn and Wilson (1948) in the telophase egg. These results suggested that cytokinesis might be taking place without the mitotic apparatus. To resolve this point, eggs were exposed to 1% urethan 5 minutes after insemination and returned to sea water 55 minutes later. Aliquots of these eggs and eggs remaining in 1% urethan were fixed 10 minutes later. The eggs that had been returned to sea water contained a normal mitotic apparatus. This is an additional confirmation of numerous observations that marine eggs reconstitute the mitotic apparatus and divide after being removed from a variety of inhibitors (Boveri, 1897; Chambers, 1938; Harvey, 1927; Hertwig, 1890; Mazia, 1959; Pease, 1941; Runnström, 1930; Wilson, 1901). The eggs left in 1% urethan contained nuclei and showed no sign of the mitotic apparatus. The nuclear cycle apparently is not inhibited by urethan.

V. Effect on the cortex

It has been proposed that the cortex may play an essential part in the mechanics of cytokinesis (Marsland, 1956; Swann and Mitchison, 1958). In addition, many of the chemical and physical agents that alter the normal physiological activities of the cell also alter the physical characteristics of the cortex (Heilbrunn, 1956). A study was therefore undertaken of the effect of urethan on the rigidity of the cortex. The method of Wilson (1951) was employed. Eggs were put into urethan 5 minutes after insemination and the cortical rigidity was determined 5 minutes later. A centrifugal force of approximately 9300 times gravity was applied to the eggs for 60 seconds by means of the hand centrifuge. An egg was considered to have a "clear" cortex when the continuous string of cortical granules was disrupted. The results of these experiments appear in Table V. In 1% urethan the eggs were almost completely devoid of cortical granules. In a small number of control eggs the continuous string of cortical granules was broken, but even in these cases a large number of granules usually remained in the cortex.

In 0.125% urethan the cortical rigidity was equal to that of the controls. At this concentration cell division and development take place normally.

The inability of the eggs to complete cytokinesis upon exposure to urethan then may result from the destruction of the mitotic apparatus and liquifaction of the cortex.

VI. Proteolytic activity during the mitotic gelation

Heilbrunn (1956) suggested that cellular sol-gel transformations, such as the mitotic gelation, are accomplished by a two-step process similar to blood coagulation: an activation stage, dependent on calcium ions, and a calcium-independent

TABLE V

The effect of urethan on cortical rigidity. Proportion of eggs exhibiting a "clear" cortex

Experiment	Control	1.0% urethan	Experiment	Control	0.125% urethan
1	3/10	9/10	1	1/10	1/10
2	1/10	8/10	2	0/10	3/10
3	3/10	8/10	3	1/10	1/10
4	0/10	7/10	4	2/10	3/10
5	3/10	7/10	5	3/10	3/10
Average	20%	78%	Average	14%	22%

Analysis of the data by means of the Chi Square test for the difference between two proportions indicates that:

1. Comparing the two control series, P is less than 0.5, therefore the difference is probably not significant.
2. Comparing the control with 1% urethan, P is less than 0.01, therefore the difference is probably very significant.
3. Comparing the control with 0.125% urethan, P is less than 0.5, therefore the difference is probably not significant.
4. Comparing 1% with 0.125% urethan, P is less than 0.01, therefore the difference is probably significant.

Eggs were exposed to urethan 5 minutes after insemination, and 5 minutes later they were exposed to a centrifugal force of approximately 9300 times gravity for 60 seconds. An egg was considered to have a "clear" cortex when the continuous string of cortical granules at the edge of the cell was disrupted.

polymerization stage, the latter presumed to be mediated by proteolytic enzymes. Gross (1954) demonstrated that the addition of ionic calcium to decalcified sea urchin egg homogenates activated a proteolytic enzyme and initiated an aggregation of nucleoproteins. Lundblad (1954) has shown that increased proteolytic activity is present in sea urchin eggs for the first 10 to 15 minutes after fertilization. This is the period when the mitotic gelation takes place in sea urchin eggs (Wilson, 1950). An attempt was therefore made to demonstrate protease activity during the mitotic gelation in *Chaetopterus* eggs, and then to determine whether urethan would affect the activation or activity of this system.

Because a considerable amount of material is necessary for an enzyme assay, the eggs of four or five ripe females were used. One aliquot of eggs was put into 1% urethan 5 minutes after insemination, and it was maintained, together with a

control, until the desired stage of the mitotic cycle was reached (25 to 32 minutes after insemination). At this point the eggs were rapidly frozen in a bath of dry ice and ethylene-glycol and were then dried under vacuum. The powder was extracted in distilled water or 0.1 *M* phosphate buffer at about 5° C. for periods of one to three hours. The extracts were centrifuged and the supernatants assayed for proteolytic activity according to the standard method of Anson (1938), which involves the colorimetric determination of acid-soluble tyrosine. Incubation periods ranged from one to five hours at 25° C.

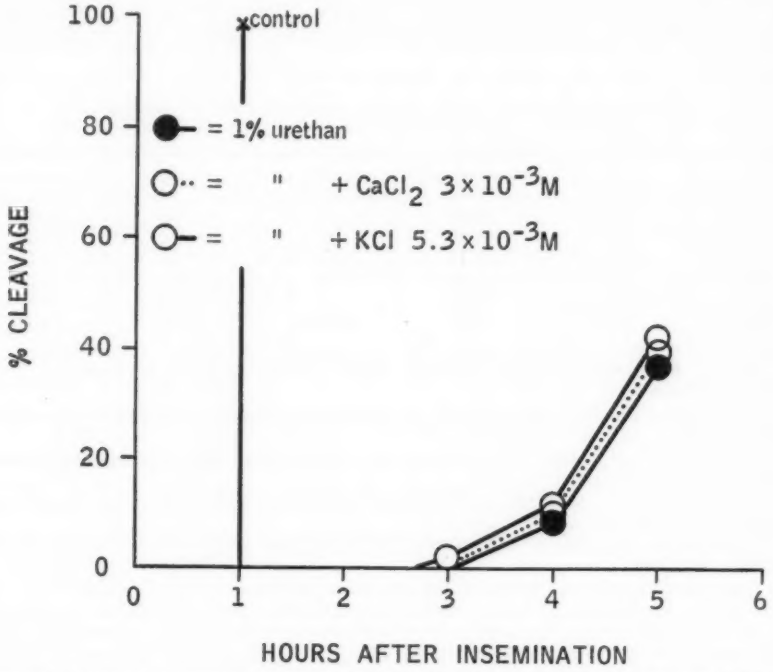

FIGURE 1. Attempt to overcome urethan inhibition of division by means of excess calcium and potassium. Eggs exposed to urethan-containing solutions 5 minutes after insemination.

The results of these experiments were completely negative. No proteolytic activity could be detected over a pH range of 3.6 to 8.0 (using acetate and phosphate buffers at 0.1 *M*) with either casein or bovine hemoglobin as substrates. In addition, tryptic digestion of casein, using Difco partially purified trypsin, was not inhibited by urethan over a concentration range of 1% to 7%.

VII. Attempts to overcome the urethan-induced inhibition of division

There is at present considerable evidence implicating multivalent cations as the "triggers" of cellular sol-gel transformations which are similar to those involved

in mitosis and cytokinesis (Anderson, 1956; Heilbrunn, 1956; Gross, 1957). It was thought that the application of multivalent cations might overcome the urethan inhibition, thus establishing the stage at which the gelation process is blocked by urethan.

The organic cations were dissolved directly in a 1% solution of urethan in sea water. Isotonic solutions of the inorganic cations containing 1% urethan were mixed with 1% urethan solutions in sea water to produce the desired concentrations.

Early experiments (Schuel, 1958) indicated that small quantities of cations

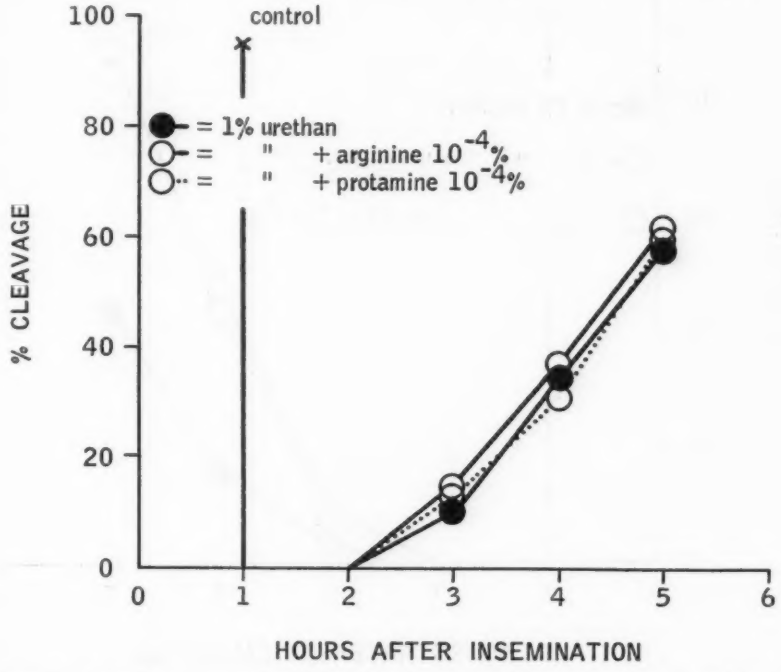

FIGURE 2. Attempt to overcome urethan inhibition of division by means of arginine and protamine. Eggs were exposed to urethan-containing solutions 5 minutes after insemination.

(inorganic calcium, histamine, protamine, arginine and lysine) could overcome the urethan inhibition. When fertilized eggs were kept in 1% urethan, very few of the eggs began to divide until about three or four hours after division had taken place in the controls. The addition of these cations made it possible for more of the eggs to divide while still in urethan. Thus, at two hours after insemination, from 10% to 30% of such eggs had already divided.

Not all of these promising results could be reproduced. In all subsequent experiments calcium, arginine and protamine failed to accelerate the recovery of eggs in urethan (Figs. 1 and 2). It was hoped that potassium might relieve the inhibition by replacing the calcium bound in the cortex, and thus liberate it for action

in the interior of the cell (Heilbrunn, 1956). This too proved to be unsuccessful (Fig. 1). In the presence of higher concentrations of potassium ions, the eggs did not divide at all. Histamine and histidine, on the other hand, did accelerate recovery. The results of a typical experiment can be seen in Figure 3.

The possible role of proteolytic enzymes in cellular sol-gel transformations has already been discussed. The addition of small quantities of chymotrypsin

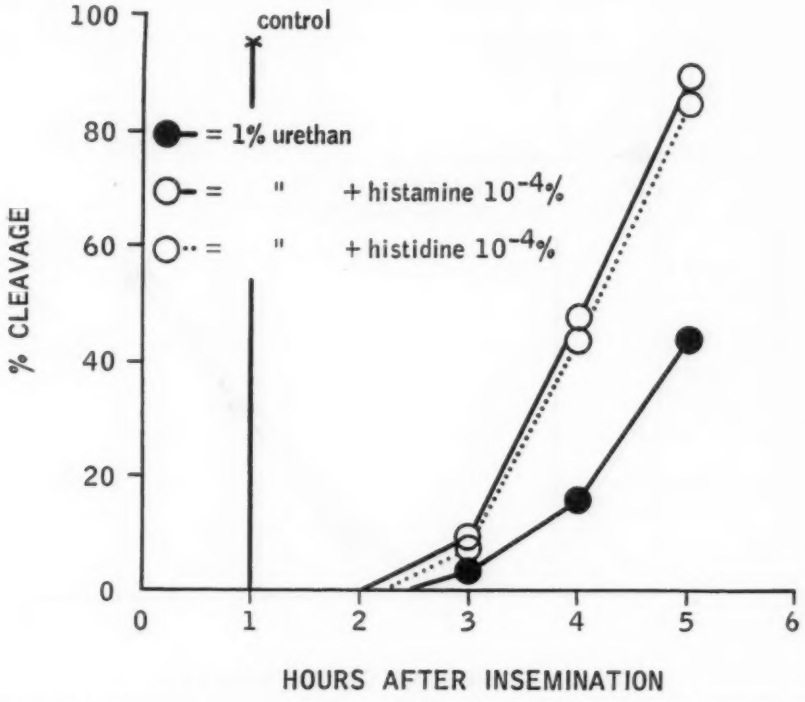

FIGURE 3. Attempt to overcome urethan inhibition of division by means of histamine and histidine. Eggs were exposed to urethan-containing solutions 5 minutes after insemination.

("Worthington 3X," crystals) or of trypsin ("Worthington lyophilized," crystals) accelerated the recovery of the eggs inhibited by 1% urethan (Fig. 4). Since calcium is believed to be bound to lipoprotein in the cortex of the cell (Heilbrunn, 1956), it was thought that lipase might also tend to relieve the urethan inhibition. Wheat germ lipase (Worthington) proved to be effective (Fig. 4). In one experiment the eggs were incubated in a mixture of trypsin and lipase (0.01 mgm./ml. each). The results were identical with those obtained by trypsin alone. However, after 24 hours the eggs were almost completely digested in the trypsin-lipase mixture, while they remained intact when incubated with either of these enzymes

alone. Ribonuclease ("Worthington 3X," crystals) had no effect on the urethan-treated eggs.

In no case, however, was there more than a temporary recovery. The eggs divided a few times to produce grape-like clusters of cells but did not develop into larvae.

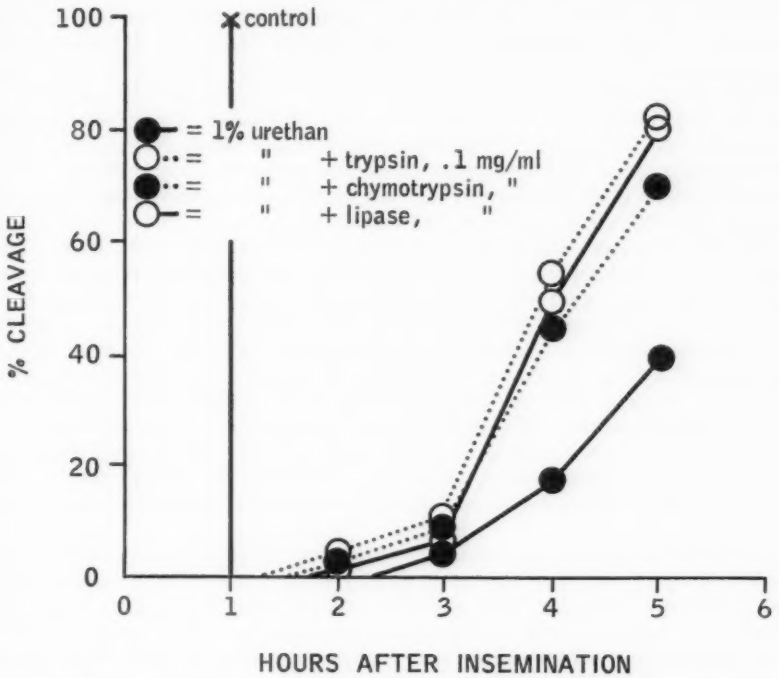

FIGURE 4. Attempt to overcome urethan inhibition of division by trypsin, chymotrypsin, and lipase. Eggs were exposed to urethan-containing solutions 5 minutes after insemination.

DISCUSSION

Several workers have attempted to explain the action of urethan as a mitotic poison in terms of an inhibition of energy metabolism (McElroy, 1947; Fisher and Henry, 1944; Warburg, 1956). Bullough (1952) suggested that the cell accumulates an energy reservoir during interphase by means of respiration and glycolysis, sufficient to maintain itself during the entire division process. Until this storage is completed the division does not begin, but once begun a division is completed at normal speed in almost any circumstance short of the death of the cell itself. Bullough's hypothesis is based, in part, on observations that the application of many physical and chemical inhibitors to cells during mitosis or cytokinesis is without effect on that division but blocks subsequent divisions.

Swann (1957) discussed this concept at considerable length. However, Gelfant (1960) has recently questioned the validity of Bullough's experiments.

Mitotic arrest can be produced at any stage of the division cycle in the *Chaetopterus* egg by the application of 1% urethan. It is unlikely that these observations could be explained in terms of a depression of respiration or an inhibition of the filling of a hypothetical energy reservoir. While it is true that sea urchin eggs are unable to maintain the organized structure of the mitotic apparatus or complete cytokinesis in the total absence of oxygen (Harvey, 1927), these eggs are able to divide under very low oxygen tensions. Inhibition of mitosis is observed only after respiration is reduced to 20% to 30% of the normal rate by very low oxygen tensions or carbon monoxide (Amberson, 1928; Krahl, 1950). Apparently when respiration is depressed to such an extent, the eggs are unable to generate enough ATP to complete the division process (Barnett, 1953). However, urethan prevents the division of these eggs at concentrations that have little effect on respiration (for references see Krahl, 1950).

The production of multipolar divisions following removal of the urethan suggests that the metabolic events preceding mitosis, chromosome duplication for example, are not the primary site of the inhibition. Apparently the eggs will divide directly into the number of cells that would have been produced by that time had there been no inhibition. Similar observations have been made by other workers (Chambers, 1938; Gross and Spindel, 1960a; Marsland, 1938; Mazia and Zimmerman, 1958; Wilson, 1901). Recently Mazia *et al.* (1960) have reported that mercaptoethanol prevents the duplication of centrioles but does not inhibit the splitting and separation of centers already duplicated, thus enabling potential poles to be realized as actual poles in multipolar divisions. A similar kind of study should be made in the future of the effects of urethan on centriole duplication and migration.

The experimental evidence indicates that urethan may be acting directly upon the mechanical structures of the dividing cell and upon the assembly of these structures. The relative cytoplasmic viscosity determinations show that urethan does not allow the mitotic gelation to take place, and the eggs cannot divide. Furthermore, the mitotic apparatus cannot be formed. It is still not clear, however, just which stage of this biochemical process is vulnerable to attack by urethan.

An attempt was made to resolve this question by means of the experiments in which cations and enzymes were used in an attempt to relieve the urethan inhibition of division. It had been suggested that either calcium, arginine or protamine might function as initiators of macromolecular aggregation reactions leading to the formation of the mitotic apparatus (Anderson, 1956; Gross, 1957; Heilbrunn, 1956). However, these materials do not relieve the block to division produced by urethan, while histidine and histamine appear to be effective in hastening division in the presence of urethan.

Heilbrunn (1956) proposed that cellular sol-gel transformations, such as the one leading to the assembly of the mitotic apparatus, might be essentially a two-step process resembling the coagulation of blood. The protease assays of the eggs were conducted in order to test this hypothesis, as well as to investigate a possible point of attack by urethan. In this case negative results cannot be considered to be conclusive. *Chaetopterus* eggs may not be the best material for these experiments since the enzyme may be active for only a very short time. Lundblad

(1954) has demonstrated the transient activity of several proteases during the first cleavage cycle in sea urchin eggs, and they might prove to be a more satisfactory material with which to study this aspect of the problem.

The application of trypsin or chymotrypsin appears to enable the eggs to divide more rapidly in the presence of urethan. Goldstein (1953) demonstrated that trypsin is able to penetrate the *Chaetopterus* egg. These enzymes may act by mediating the second stage of the aggregation reaction within the eggs, and thus enable mitosis to continue. Alternatively, they might digest some intracellular proteins, thus releasing large amounts of organic or inorganic cations. Lipase might act by attacking the lipo-protein complex which is thought to bind calcium in the cortex (Heilbrunn, 1956).

In this connection the experiments concerning the reversal of the urethan inhibition on return to sea water are very suggestive. The eggs readily re-form the mitotic apparatus prior to division. Moreover the changes in cytoplasmic viscosity during the recovery period are similar to those observed in cytokinesis. Apparently the system is primed and ready to complete division in the presence of urethan, and does so as soon as the inhibitor is removed. Urethan thus may be affecting some chemical bonds that are essential for the polymerization of the spindle proteins.

If this is true, one might also expect urethan to destroy the mitotic apparatus after it has been formed. The cytological observations reported above show that this is indeed the situation. The mitotic apparatus is a very labile structure, and is readily dispersed by mechanical agitation (Chambers, 1938; Wilson, 1901), hydrostatic pressure (Pease, 1941), temperature shocks (Boveri, 1897) and narcotics (Swann, 1954; Wilson, 1901). These observations suggest that some kind of relatively weak chemical bond may play an important role in the maintenance of the organized structure of the mitotic apparatus.

A possible clue to the chemical mechanisms involved may be found in the recent experiments with heavy water and marine eggs (Gross and Spindel, 1960a, 1960b). Deuterium oxide inhibited division at any stage of the mitotic cycle prior to the completion of cytokinesis. The eggs in heavy water were all arrested in the stage of entry, and a gelation of the cytoplasm was also observed. These are the results one would expect with a primarily hydrogen-bonded structure. The urethan effects that have been found in this study (prevention of the mitotic gelation, prevention of the assembly of the mitotic apparatus, dispersion of the mitotic apparatus after it had been formed) may be reflections of an interaction with the hydrogen bonds that bind together the macromolecular components of the mitotic apparatus. In this connection, observations indicating that urethan may rupture hydrogen bonds in proteins are very suggestive (Johnson *et al.*, 1948).

Additional work is necessary before the exact chemical mechanism of the urethan inhibition of division in marine eggs can be established. Moreover, it would be desirable to know whether similar reactions are responsible for the inhibition of cell division in mammalian tissues and tumors.

Summary

1. Dilute solutions of urethan (about 1%) prevent the division of fertilized *Chaetopterus* eggs when the eggs are treated beginning 5 minutes after insemination.

Relative cytoplasmic viscosity determinations show that the mitotic gelation, which immediately precedes the formation of the mitotic apparatus, does not take place. In concentrations of urethan insufficient to prevent division (less than 0.5%) the mitotic gelation occurs normally. The inhibition is reversible since the eggs reassemble the mitotic apparatus and divide following return to normal sea water. Upon return to sea water the inhibited eggs divide directly into the number of cells present in the controls at that time, thus indicating that metabolic work and chromosome duplication are not affected. During this period there is a rapid and sharp increase in relative cytoplasmic viscosity. Eggs that have begun to form cleavage furrows are not able to complete cytokinesis upon exposure to urethan. The rigidity of the cortex is reduced by inhibitory concentrations of urethan. Cytological preparations reveal that urethan prevents the formation of the mitotic apparatus, and also destroys the organized structure of the mitotic apparatus after it has been formed.

2. Few of the eggs in 1% urethan divide until about four or five hours after fertilization. Untreated eggs at 21°C. divide by 58 minutes after insemination. The addition of small quantities of histamine, histidine, trypsin, chymotrypsin or lipase enables the eggs to divide sooner than four to five hours in the presence of urethan. Calcium, potassium, arginine and protamine were not effective.

3. It has been proposed that the urethan effects observed in this investigation may be reflections of an interaction with the hydrogen bonds that are believed to link together the macromolecular components of the mitotic apparatus.

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A BLOOD GROUP SYSTEM FOR SPINY DOGFISH, SQUALUS ACANTHIAS L.

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Recent expansion of interest in fish immunogenetics, particularly in blood group antigens (Cushing and Durall, 1957; Ridgway, Cushing and Durall, 1958; Suzuki, Shimizu and Morio, 1958; Sindermann and Mairs, 1959; Ridgway and Klontz, 1960), has resulted in description of antigen systems for tuna and herring, and in quantitative information about antigen frequencies in populations of herring, tuna and salmon. The elasmobranch fishes have not been examined thus far, but it seems quite possible that serological methods of population and migration study could be of particular value to investigations of certain shark species. The recognition and description of blood group antigens, and where possible the genetic systems that determine their occurrence, are preludes to quantitative determinations of antigen and gene frequencies in populations. As an exploratory step in such studies of sharks, and as part of a general immunogenetic investigation of marine organisms, this report is concerned with isoagglutinins and erythrocyte antigens of the spiny dogfish, *Squalus acanthias* L. Included are serological approaches and data supporting the proposal of a first blood group system—the "S" system—for this species.

Materials and Methods

Dogfish were obtained from several places in the Gulf of Maine (Georges Bank, Casco Bay, Sheepscot Bay and Muscongus Bay) and were bled by puncturing the heart or by severing the caudal artery. Serum was decanted after overnight expression at 4° C. and inactivated at 50° C. for 30 minutes to destroy complement. Cells were washed three times in 1.5% saline and used in approximately 4% suspensions in 1.5% saline. Cross-reactions among dogfish sera and cells were tested by tube agglutination with .2 ml. undiluted serum and .05 ml. cell suspension. Tubes were read after 30-minute incubation at room temperature. Presence and degree of agglutination were recorded conventionally with the following symbols: ++++, complete agglutination with a single consolidated pellet; +++, strong agglutination with several large clumps; ++, moderate agglutination with numerous smaller clumps; +, weak agglutination with many very small clumps visible macroscopically; −, no agglutination. Saline controls were used throughout, and critical tests repeated. Absorptions of dogfish sera—which may remove certain antibodies selectively—involved addition of undiluted serum from one fish to packed washed cells from another fish in 1 : 1 proportions. The cell-serum sus-

[1] The authors would like to acknowledge the technical assistance of Alva Farrin, U. S. Bureau of Commercial Fisheries, Boothbay Harbor, Maine. The Casco Bay dogfish-processing laboratory of Wards Natural Science Establishment, and the Maine Department of Sea and Shore Fisheries cooperated in obtaining some of the blood samples used in this study.

pension was incubated 10 minutes at room temperature, centrifuged, and the absorbed serum decanted and used in cell agglutination tests. One such absorption was sufficient to remove all antibodies reactive with the absorbing cells but in some cases left antibodies for cells from other fish.

Rabbit antisera were produced by 6 subcutaneous injections of 1 ml. washed dogfish cells given on alternate days. The rabbits were bled terminally 10 days after the last injection, and the antisera frozen in 3-ml. aliquots until used. Antisera were inactivated at 56° C. for 30 minutes just before testing, to destroy complement. Tests by tube agglutination used .2 ml. diluted antiserum and .05 ml. cell suspension. Readings were taken after 15-minute incubation at room temperature and 30-second centrifugation. Presence and degree of agglutination were recorded as described above. Antiserum absorptions, which constitute a fractionation method to produce specific reagents, involved addition of antiserum (diluted 1:2 with 1.5% saline) to packed washed cells from individual dogfish in proportions of 1 part cells to 4 parts antiserum. The cell-antiserum suspension was incubated 10 minutes at room temperature, centrifuged, and the absorbed antiserum decanted and tested at 1:4 dilution. One such absorption was sufficient to remove all antibodies reacting with the absorbing cells but in some cases left antibodies capable of reacting with cells from other individuals.

Dogfish donors and recipients used in isoimmunizations were tagged and maintained in sea-water tanks of the U. S. Bureau of Commercial Fisheries Biological Laboratory at Boothbay Harbor. The fish were trial bled by heart puncture; cross-reactions and tests with rabbit antisera were carried out on the samples obtained. Results of these tests determined the nature of the subsequent immunizations attempted. Recipient dogfish received 2 ml. of cells bled from the donor weekly and washed three times before injections, which were alternately intraperitoneal and intracardial. Trial bleedings for isoimmune antibodies were made two weeks following the fourth injection.

Dogfish embryos (15–23 cm.) used in the comparative study of erythrocyte antigens were obtained by dissections of gravid females or were aborted by such females after capture. They were bled by severing the caudal artery or by heart incision and tested with the same methods used for adults.

Results

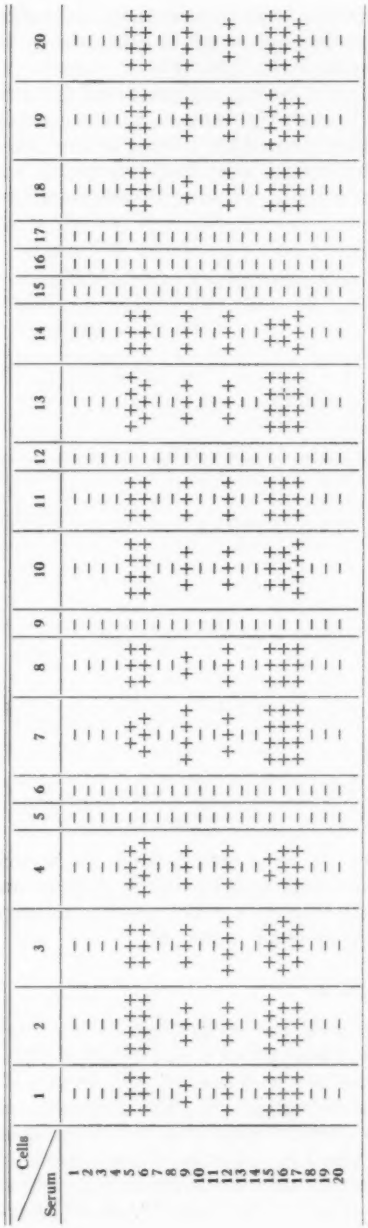
1. *Isoagglutination*

Cross-matching of cells and undiluted sera from 20 individuals gave the first clear indication of individual antigenic differences in dogfish, as seen in Table I. Sera from fish numbered 5, 6, 9, 12, 15, 16 and 17 contained isoantibodies (isoagglutinins) that reacted with cells of fish numbered 1, 2, 3, 4, 7, 8, 10, 11, 13, 14, 18, 19 and 20. Sera from fish whose cells were agglutinated did not contain antibodies for any of the cells tested. Conversely, cells from fish whose serum contained the agglutinins did not possess antigens reacting with any of the sera tested. The average isoagglutinin titer of the sera tested was 4.

The simplest explanation for the observed results was that the positive cells contained an antigen (tentatively labelled "antigen S") which negative cells lacked and that agglutinating sera contained a specific "anti-S" agglutinin for the antigen.

TABLE 1

Cross-reactions of sera and cells from 20 dogfish



Validity of this explanation was supported by absorptions of sera containing anti-S agglutinins with positive and negative cells, and by an absorption analysis of a single serum, as illustrated in Table II.

Absorptions with cells lacking antigen S did not remove any antibodies, while absorptions with cells carrying antigen S removed all antibodies, not only for themselves, but for all other positive cells as well. This is strong evidence that the antigens involved are identical, or at least very nearly so. Sera containing the anti-S agglutinin were pooled as a test reagent (Reagent 1) for quantitative studies of

TABLE II

Results of absorption of dogfish isoantibodies by dogfish cells

Serum	Absorbed with cells of	Cells tested									
		1	2	5	6	9	16	17	18	19	20
5	1	–	–	–	–	–	–	–	–	–	–
5	2	–	–	–	–	–	–	–	–	–	–
5	5	+++	+++	–	–	–	–	–	+++	++++	+++
5	6	+++	++++	–	–	–	–	–	+++	+++	+++
5	9	+++	+++	–	–	–	–	–	++++	++	+++
5	16	+++	+++	–	–	–	–	–	+++	+++	++++
5	17	+++	++	–	–	–	–	–	++	+++	+++
5	18	–	–	–	–	–	–	–	–	–	–
5	19	–	–	–	–	–	–	–	–	–	–
5	20	–	–	–	–	–	–	–	–	–	–
6	5	+++	++++	–	–	–	–	–	++	+++	+++
6	1	–	–	–	–	–	–	–	–	–	–
9	2	–	–	–	–	–	–	–	–	–	–
12	9	+++	+++	–	–	–	–	–	++	+++	+++
15	17	+++	++++	–	–	–	–	–	+++	++++	++
16	18	–	–	–	–	–	–	–	–	–	–
17	5	+++	++	–	–	–	–	–	+++	++++	++++
18	2	–	–	–	–	–	–	–	–	–	–
18	16	–	–	–	–	–	–	–	–	–	–

S-antigen frequencies, as were sera lacking the anti-S agglutinin (Reagent 2). All subsequent samples were tested with the reagents and were also cross-matched in groups of 10 or 20, since it was not possible to keep cells for longer than one week without freezing or other treatment.

2. *Antiserum absorptions*

Further elucidation of the antigen system disclosed by isoagglutination was obtained by absorption analysis of a rabbit anti-dogfish serum (BHD64R). The rabbit had been immunized with washed cells from a single dogfish found in preliminary tests to possess antigen S. The antibody content of the normal serum of this rabbit before immunization was not determined, but the titer of the unabsorbed antiserum was 128. Results of absorptions of this antiserum with cells of the same dogfish used in the isoagglutination study are illustrated in Table III.

Absorption with cells of 5, 6, 9 and 12, already found to lack reactive antigens

in cross-matching tests, removed species antibodies—those which indiscriminately agglutinate cells of all individuals—but left antibodies which agglutinated cells of 1, 2, 3, 4, 7, 8, 10 and 11. Results of tests with the absorbed antiserum agreed with those obtained in the isoagglutinin study, cells of individuals 1, 2, 3, 4, 7, 8, 10, and 11 carrying an antigen not occurring on cells of individuals 5, 6, 9, and 12. The absorptions further disclosed a second antigen on the cells of fish number 2 that was not detected by isoantibodies in dogfish sera. Absorption of the rabbit antiserum with cells of dogfish number 2 removed antibodies for all other cells, while absorption with cells of 1, 3, 4, 7, 8, 10 and 11 removed antibodies for all others except those of fish number 2. For descriptive purposes the antigens detected by absorbed rabbit antiserum have been labelled "S_1" and "S_2"—S_1 being identical to the antigen detected by isoagglutination. Both antigens were reactive with the reagent prepared by pooling antiserum absorbed by cells of 5, 6, 9, and

TABLE III

Results of absorptions of rabbit anti-dogfish serum by dogfish cells

Antiserum BHD64R absorbed with cells of dogfish:	Test cells											
	1	2	3	4	5	6	7	8	9	10	11	12
1	−	++	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−
3	−	++	−	−	−	−	−	−	−	−	−	−
4	−	++	−	−	−	−	−	−	−	−	−	−
5	+	++	+	+	−	−	+	+	−	+	+	−
6	+	++	+	+	−	−	+	+	−	+	+	−
7	−	++	−	−	−	−	−	−	−	−	−	−
8	−	++	−	−	−	−	−	−	−	−	−	−
9	+	++	+	+	−	−	+	+	−	+	+	−
10	−	++	−	−	−	−	−	−	−	−	−	−
11	−	++	−	−	−	−	−	−	−	−	−	−
12	+	++	+	+	−	−	+	+	−	+	+	−
Unabsorbed	+++	+++	+++	++++	++	++	++++	++++	+++	++++	+++	++

12 (Reagent 3), and S_2 was reactive with the reagent prepared by pooling antiserum absorbed with cells of 1, 3, 4, 7, 8, 10 and 11 (Reagent 4). Antiserum absorbed with cells of fish number 2 constituted a negative control reagent (Reagent 5).

According to the reactions obtained, fish number 2 possessed antigens S_1 and S_2, fish number 1, 3, 4, 7, 8, 10 and 11 possessed antigen S_1, and fish number 5, 6, 9 and 12 lacked both antigens.

3. *Definition of reagents*

The results of isoagglutinations and antisera absorptions presented in previous sections show that it is possible to define five reagents for the detection of antigenic specificities S_1 and S_2.

(1) Dogfish serum containing anti-S agglutinins specific for S_1 antigen.
(2) Dogfish serum from S_1 positive fish lacking anti-S agglutinins—a negative control.
(3) Absorbed rabbit anti-dogfish serum (BHD64R) containing antibodies specific for S_1 and S_2 antigens.

TABLE IV

*Dogfish isoagglutination and antiserum absorptions**

Reagent:	Test cells									
	21 (S_9)	22 (S_1)	23 (S_3)	24 (S_9)	25 (S_4)	26 (S_1)	27 (S_1)	28 (S_2)	29 (S_4)	30 (S_1)
(1) Dogfish anti-S_1	−	++++	+++	−	++++	++++	+++	−	++++	++++
(2) Dogfish negative	−	−	−	−	−	−	−	−	−	−
(3) Rabbit anti-S_1 & S_2	−	+++	++	−	++	+	++	++	+	++
(4) Rabbit anti-S_2	−	−	−	−	−	−	−	++	−	−
(5) Rabbit negative	−	−	−	−	−	−	−	−	−	−
Serum of Dogfish No.										
21 (anti-S_1)	−	+++	+	−	++	++	+	−	++	+++
22 (−)	−	−	−	−	−	−	−	−	−	−
23 (−)	−	−	−	−	−	−	−	−	−	−
24 (anti-S_1)	−	++++	++	−	++	++	++++	−	++	++
25 (−)	−	−	−	−	−	−	−	−	−	−
26 (−)	−	−	−	−	−	−	−	−	−	−
27 (−)	−	−	−	−	−	−	−	−	−	−
28 (anti-S_1)	−	++++	++++	−	+++	++++	++++	−	++++	+++
29 (−)	−	−	−	−	−	−	−	−	−	−
30 (−)	−	−	−	−	−	−	−	−	−	−
Antiserum BHD64R absorbed with cells of dogfish										
21 (S_9)	−	+	++	−	+	++	+	+	++	+
22 (S_1)	−	−	−	−	−	−	−	++	−	−
23 (S_1)	−	−	−	−	−	−	−	++	−	−
24 (S_9)	−	+	+	−	+	+	+	++	+	+
25 (S_1)	−	−	−	−	−	−	−	++	−	−
26 (S_1)	−	−	−	−	−	−	−	++	−	−
27 (S_1)	−	−	−	−	−	−	−	+	−	−
28 (S_2)	−	+	++	−	++	++	++	−	++	+
29 (S_4)	−	−	−	−	−	−	−	+	−	−
30 (S_1)	−	−	−	−	−	−	−	+	−	−
Unabsorbed	++	++++	+++	+++	++++	++++	+++	++++	+++	+++

* Erythrocyte antigens of each fish, as disclosed by this analysis, are in parentheses after each test cell number.

(4) Absorbed rabbit anti-dogfish serum (BHD64R) containing antibodies specific for S_2 antigen.
(5) Absorbed rabbit anti-dogfish serum (BHD64R) lacking antibodies for S_1 and S_2—a negative control.

The reality of the two antigens in the system and the specificity of the reagents for their detection were clearly demonstrated with a second series of samples tested first with the five reagents, then examined for isoagglutination, and finally analyzed by antiserum absorption, as seen in Table IV.

From this analysis, dogfish 22, 23, 25, 26, 27, 29 and 30 possessed S_1 antigen, 28 possessed S_2 antigen, 21 and 24 lacked either antigen, and none had both antigens. The antiserum absorptions provided a sixth reagent (absorbed with S_2 cells of 28) that was specific for cells containing S_1 antigen—completing the battery of reagents prepared from antiserum BHD64R necessary for detection of both S_1 and S_2 in quantitative studies of antigen frequencies. Subsequent samples, totalling 138, were tested with all six reagents. Dogfish 28 was the only one found to carry only antigen S_2.

4. *Isoimmunization attempts*

A further analysis of the erythrocyte antigens of individual dogfish was attempted with isoimmunization—injecting the cells of one fish into another fish. Dogfish in the series 21 to 30 described in the previous section and already characterized according to S antigens, were tagged and maintained in the sea-water tanks of the U. S. Bureau of Commercial Fisheries Biological Laboratory at Boothbay Harbor and were used in isoimmunization attempts, according to the following experimental design:

(1) dogfish 21, which lacked S_1 antigen and contained isoagglutinins for S_1, was injected with washed cells of 30 which possessed S_1;
(2) dogfish 25, which possessed S_1 antigen and lacked isoagglutinins for S_1, was injected with cells of 22, which possessed S_1; and
(3) dogfish 26 which possessed S_1 antigen and lacked isoagglutinins for S_1, was injected with cells of 24, which lacked S_1.

Two weeks after the final injection, sera from the three recipient dogfish were tested against cells of donors, and against cells of other dogfish in the series 21 to 30.

No change in agglutinin titer or specificity of dogfish sera was found after the isoimmunization attempts. Only the reactions of antigen S_1 were tested, since the single S_2 dogfish (number 28) died shortly after the first test bleeding. Under the conditions of the experiment (four weekly injections, tests two weeks after the last injection, sea-water temperature 12–17° C.) injection of S_1 cells into a recipient lacking S_1 caused no increase in anti-S_1 agglutinin titer. Injection of S_0 cells into a S_1 individual did not produce anti-S_0 agglutinins. Further isoimmunizations should be attempted with dogfish possessing S_2 and other antigens, possibly extending over a greater period of time or involving more frequent injections.

5. *Comparison of erythrocyte antigens of gravid females and embryos*

Since the spiny dogfish is an ovoviviparous species, it was possible to obtain presumptive genetic information about the S blood groups by comparing erythrocyte

* Erythrocyte antigens of each fish, as disclosed by this analysis, are in parentheses after each test cell number.

antigens of females and their well-developed embryos ("pups"). Detectable isoantibodies were not present in sera of nearly full-term pups, but erythrocyte antigens—agglutinated by isoantibodies in sera from adult dogfish and by absorbed antiserum—gave reactions of the same specificity and intensity as those of adult dogfish. Blood groups of females and their progeny are presented in Table V. Females with blood group S_1 had S_1S_2, S_1 and S_0 pups, while females with blood group S_0 had S_0 and S_1 pups.

TABLE V

Blood groups of gravid female dogfish and their unborn progeny

Blood group of mother	Blood group of embryos
(1) S_1	S_1, S_1, S_1, S_1, S_0
(2) S_0	S_1, S_1, S_1, S_1, S_1
(3) S_1	S_1S_2, S_0, S_0
(4) S_0	S_1, S_1, S_1, S_1
(5) S_1	S_1, S_1, S_1
(6) S_0	S_1, S_0, S_0, S_0
(7) S_1	S_1, S_1, S_1, S_1, S_0
(8) S_0	S_1, S_1, S_0, S_0, S_0, S_0, S_0, S_0
(9) S_1	S_1, S_1, S_1, S_1
(10) S_0	S_0, S_0, S_0, S_0, S_0
(11) S_1	S_1, S_1
(12) S_1	S_1, S_1, S_1, S_1, S_1, S_0

6. *The S blood group system of dogfish*

Although further genetic evidence—preferably based on results of known crosses—is necessary, the S blood group system can be proposed tentatively. Based on (1) results of isoagglutinations, antiserum absorptions and isoimmunization attempts; (2) comparisons of maternal blood groups with those of unborn pups; and (3) analyses of 138 individual dogfish blood samples from different Gulf of Maine areas with the six reagents described earlier, the following statements can be made:

(1) The S blood group system of spiny dogfish, *Squalus acanthias*, contains at least two antigens, S_1 and S_2, that may or may not be possessed by individual fish.

(2) Isoantibodies present in sera of most dogfish whose cells lack S_1 antigen will agglutinate cells possessing only S_1. Isoagglutinins for S_2 antigens have not been detected.

(3) Antigen S_1 can be demonstrated with antibodies remaining in rabbit antiserum after absorption by cells of dogfish containing S_2 antigen, and antigen S_2 with antibodies remaining after absorption by S_1 cells. Cells not agglutinated by either absorbed antiserum have been designated S_0. Thus far an anti-S_0 serum has not been found.

(4) Individual fish may possess both, either, or neither of the antigens, so possible blood groups (types) are S_1S_2, S_1, S_2, and S_0.

(5) Of the 138 dogfish tested thus far from the Gulf of Maine, two individuals had S_1S_2 blood group, 92 had S_1, 1 had S_2 and 43 had S_0.

Discussion

The present paper demonstrates individual differences in erythrocyte antigens and an isoantibody system in a species of the elasmobranch fishes. A clearly defined antigen system, demonstrable with isoagglutinins and absorbed rabbit antiserum, exists in spiny dogfish. The system parallels the Tg system of tuna (Suzuki, Shimizu and Morio, 1958) in that it is composed of two antigens, with individuals possessing either, both or neither antigen. Similarities also exist with an isoagglutinin system described by Cushing and Durall (1957) in brown bullheads (*Ictalurus n. nebulosus*). The dogfish blood groups described here seem definitive enough in their present state to be used in quantitative studies of populations and in tracing migrations of infraspecies groups, if geographic variations in antigen frequencies exist. Thus far six individuals have been found that lacked S antigens and also lacked detectable isoagglutinins in their serum. This might be expected, since the agglutinin titers of individual sera varied considerably. As greater numbers of samples are tested, exceptions may occur, as they have been found to occur in human and other animal groups (Owen, 1954; Stormont, 1955), but the proposed system has proved remarkably specific and definitive thus far in tests of *Squalus* from the Gulf of Maine. Future studies may suggest modification of the system, just as extended studies of human blood group systems have resulted in modifications of original hypotheses (Landsteiner, 1901; von Dungern and Hirzsfeld, 1911; Friedenreich, 1936), but the reality of the dogfish S antigens described here seems clear.

Blood group systems are essentially genetic systems, and hence cannot be fully described, understood or accepted without genetic information. Any postulated classification of individual antigenic differences—such as that here proposed for dogfish—should be supported eventually by genetic evidence. It is difficult to make experimental crosses and to rear offspring of most marine fish for such studies, but the ovoviviparous species including *Squalus* can provide some interim data. Comparing blood groups of pregnant females and their unborn offspring may offer presumptive genetic evidence supporting erection of blood group systems. Such a comparison of female dogfish and their pups in the present work suggests that the S system of blood group antigens may be controlled by three alleles: S^1, S^2 and S^0. According to this explanation the S_1S_2 phenotype would have S^1S^2 genotype; S_1 would have S^1S^1 or S^1S^0; S_2 would have S^2S^2 or S^2S^0; and S_0 would have S^0S^0. Based on a three-allele hypothesis, quantitative data from the limited sample of adult dogfish presented in this paper result in the following gene frequencies: S^1—0.44, S^2—0.01, S^0—0.55. The number of individuals tested is small, so expected ideal values for the entire Gulf of Maine population may not be represented. Also, there may be local differences in frequencies, so the next logical step would be determination of antigen and gene frequencies in large samples of *Squalus* from different geographic areas. In addition to testing the genetic hypothesis, this quantitative work could provide some information about the degree of reproductive isolation of groups and the subpopulation structure of the species.

The existence of sharp individual differences in the antigenic constitution of dogfish offers the promise of comparable differences in other elasmobranch fishes. Since certain of the larger shark species are receiving increasing attention from biologists as well as from the popular press (Gilbert, 1960), immunogenetic methods

may offer a useful approach to studies of their migrations and populations. While the large sharks provide a practical stimulus for research, smaller selachians such as dogfish may actually be animals of choice in such investigations. In addition to the possession of a clearly defined isoantibody system, dogfish occur in schools, are easily handled in marine aquaria, and yield copious amounts of blood. Also, since they are ovoviviparous, some genetic information may be acquired by comparing erythrocyte antigens of females and their pups. Future research in this laboratory will extend the comparative studies already reported, will include quantitative sampling for S antigen frequencies in different geographic areas, and will extend to other elasmobranch species—particularly the larger sharks.

Summary

1. An erythrocyte antigen system of dogfish, *Squalus acanthias*, has been recognized and described with a progression of serological procedures including isoagglutination, antiserum absorption and isoimmunization. This system, the "S" blood group system, contains at least two antigens, "S_1" and "S_2." Individual fish may possess both, either or neither of the antigens, being of blood groups (types) S_1S_2, S_1, S_2 or S_0, respectively. Reagents specific for the antigens have been prepared from normal dogfish sera and absorbed rabbit antiserum, for quantitative studies of blood group frequencies in dogfish from different geographic areas. Fish sampled thus far in the Gulf of Maine (138 individuals) have the following frequencies: S_1S_2—1.4%, S_1—66.7%, S_2—0.7%, and S_0—31.2%. Blood groups of females and unborn pups are compatible with a simple genetic hypothesis of three alleles, S^1, S^2 and S^0, controlling the system.

2. The definitive nature of this system in *Squalus* promises potentially profitable immunogenetic investigations of other elasmobranchs, including the larger shark species. Blood group frequencies can be used in determining the degree of reproductive isolation of populations and may provide information about migrations of fishes.

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IONIC GRADIENTS IN SOME INVERTEBRATE SPERMATOZOA [1]

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While the variety of male gametes seems almost infinite, the vast majority of animals produce a spermatozoan of fairly typical structure consisting of a head, midpiece and tail (Retzius, 1910). The indications from many different sources are that these three regions (plus other specialized areas such as the acrosome) have highly specific functions. The midpiece, with its mitochondrial structure, is implicated in general metabolism, the head is heavy with genetic information via DNA, and the tail functions as a locomotor organelle to propel the information unit towards its ultimate repository, *i.e.*, the ovum or death (*cf.* Mann, 1949).

Compartmentalized as it is in neat morphological packages, the typical spermatozoan becomes a tempting material with which to attempt to identify various bits of chemical machinery ubiquitous in all cells. A fairly extensive body of literature exists on the chemical and metabolic structure of the head and, to a lesser extent, on the midpiece. Isolated tail fractions have been shown to contain special fibrous proteins which may contribute the contractile properties (*cf.* Nelson, 1959), some of these components being arranged according to a precise numerology that awaits final elucidation (*cf.* Serra, 1960).

One of the most characteristic features of living cells, neglecting for the moment bacteria, etc., is the existence of an ionic gradient $K_i > K_o$ and, for most cells $Na_o > Na_i$ (Steinbach, 1952). These gradients are generally associated with irritability of cells and have at times been discussed as a reflection, at least, of an available free energy gradient (Fleckenstein, 1954). Ionic gradients have not been studied in any detail in sperm cells and, so far as we are aware, no previous attempt has been made to determine whether the production and/or the maintenance of an ionic gradient is a function of the whole structure or only a part thereof. The present report presents preliminary data on the distribution and exchangeability of Na and K of whole sperm and of separated head-midpiece fractions and tails. The head-midpiece complex, for the sperm of three species tested, appears capable of maintaining good ionic gradients. Tails, separated from the rest of the complex, appear to contain Na, K and Cl in free diffusion equilibrium with the environment.

Material and Methods

Mature sperm were collected as follows:

1. Male *Arbacia punctulata* were induced to shed into sea water by electrical stimulation. The suspensions were then diluted appropriately and used as described below.

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[2] Trainee in United States Public Health Service Training Program 2G-150.

2. *Mytilus edulis* sperm were collected by mincing the gonads in sea water, straining the resultant brei through cheesecloth and then subjecting to mild centrifugation to remove heavier contaminants. Microscopic examination showed these preparations to be reasonably free of tissue fragments.
3. *Phascolosoma gouldi* sperm were collected by draining body fluids of mature males into sea water and removing the large cell components by mild centrifuging. Clean suspensions of sperm could be obtained easily in this fashion.

The general pattern of treatment of sperm suspensions was as follows: The suspensions, maintained at sea-water table temperature (18–22° C.), were adjusted to the desired chemical composition. Separation of head-midpieces from tails was accomplished by 5–10 seconds at high speed in a Servall Blender. This method gave suspensions that could be separated by 10 minutes' centrifuging at ca. 2000 G into a pellet of apparently tail-less head-midpiece structures and a cloudy supernatant of filamentous tails. Especially in Mytilus, the tail suspension fibers frequently aggregated into sheaves.

Tails were collected as pellets by centrifuging the supernatant of the low speed centrifugation for 10 minutes at 15,000 G in a Servall superspeed centrifuge. This resulted in a clear supernatant apparently devoid of formed elements.

Concentration of cells was adjusted so that 10 ml. of suspension of whole sperm yielded on the order of 100–200 mg. of packed pellet as determined by weighing previously tared centrifuge tubes. Rather than attempt extensive cell counts, estimates of trapped fluid in the pellets were made by adding either C^{14} inulin to the suspension or $Na_2S^{35}O_4$ thus enabling the determination of inulin or sulphate "spaces." As noted below, there is certainly some question as to whether this is a true measure of a morphological volume of extracellular or cellular space, but the method seems more meaningful than the highly variable cell count method of determining cell volume. With fractions of such varying chemical composition it was deemed unwise to attempt to express Na or K contents per unit of nitrogen or of dry weight. Thus concentrations are expressed as millimols per 1000 grams of wet pellet or as figures derived from these on the basis of inulin spaces and/or dry weights.

Na and K were measured on extracts of pellets or on supernatant fluids with the Coleman flame photometer. Chlorides were frequently measured on the same solutions with an Aminco-Cotlove chloride meter. Extracts were prepared by heating briefly the suspended pellet in 10 ml. of water with 2 drops of glacial acetic acid added. Digestion with HNO_3 of residues from the above treatment showed almost complete removal of Na, K and Cl from the suspended material.

C^{14} was counted in the usual manner with a thin window gas flow counter.

Results

Table I summarizes the results of the first series of observations on the basic composition of the three types of sperm. Figures for bull and human sperm, uncorrected for inulin spaces, are given for comparison. Inulin spaces of the pellets are high and there are reasons to believe that the inulin is penetrating into some portions of the cell mass. However, inulin space is not greatly variable with time and is used in calculating cell water concentrations. Several points need

emphasis. K is concentrated in the sperm as it is in other cellular systems. If cell water volume could be truly represented as total pellet volume minus dry weight and inulin space, the K concentration of cell water is very high indeed for Arbacia and Phascolosoma.

Na and Cl contents are variable, as will be apparent also from subsequent tables. Table I indicates that no chloride is present in the non-inulin space. On the contrary, the fact that inulin space frequently exceeds chloride space of the pellet is an indication that inulin penetrates to some extent within the morphological boundaries of the sperm.

TABLE I

Dry weights and inulin spaces (as % of wet pellet) and concentrations as mM./1000 grams of wet pellets. Derived concentrations (mM./L. cell water) calculated assuming 97% of dry weight of pellet to be in sperm and external medium of composition Na = 410, Cl = 500, K = 10 mM. Composition of bull and human sperm from Cragle et al., 1958 and Keitel and Jones, 1956.

	% Dry (pellet)	Inulin %	% Dry (sperm)	Na		Cl		K	
				Pellet	$Cell_{H_2O}$	Pellet	$Cell_{H_2O}$	Pellet	$Cell_{H_2O}$
Arbacia (2)	24	46	45	195	23	150	—	136	440
Mytilus (4)	27	26	36	166	107	161	—	78	145
Phascolosoma (8)	18	44	32	209	76	216	—	115	300
Bull				76				62	
Human			16	101		28		39	

It is known that sperm of such forms as Arbacia and Mytilus can survive, properly diluted in sea water, for periods of several hours. Table II shows that these sperm can also maintain their ionic gradients for extended periods of time. With Mytilus there is a decrease of pellet size with time. In the absence of counts of viable cells, it is impossible to tell whether this involves an average decrease in cell size or a disintegration of some fraction of the populations. Whichever the cause, the decrease takes place within the first hour to hour and a half, pellet size being constant thereafter.

Potassium in sperm is nearly completely exchangeable (Fig. 1) at a fairly rapid rate. Half-time for exchange of cellular K with K^{42} of the medium is on the order of three hours for Mytilus, 1.5 hours for Phascolosoma, and less than an hour for Arbacia.

Figure 1 shows that the time course of exchange of K^{42} added to the medium with K of the pellet follows the usual course of an initial rapid exchange (extracellular?) followed by a second slower component. There is, however, no indication that any significant amount of K is sequestered within the sperm or otherwise made inaccessible to free exchange. The exchange of Na^{24} with Na of sperm is very rapid. Accurate results have not been obtained but the half-time for exchange is distinctly less than 30 minutes for all three types of sperm.

Table III lists average values for the analyses of head-midpiece and tail fractions as compared to whole sperm from the same species. The values listed

TABLE II

Pellet concentrations (mM./1000 gm. wet) of Na, Cl and K for samples of a suspension centrifuged at the times indicated. Sperm suspensions stored on sea water table at 20° C.

Time minutes	Weight of pellet %	Conc. mM./Kg.		
		Na	Cl	K
Mytilus				
10	100	162	126	74
30	98	159	164	75
90	91	155	151	67
210	90	155	139	64
Arbacia				
20	100	193	287	110
130	101	168	267	88
300	100	174	232	99

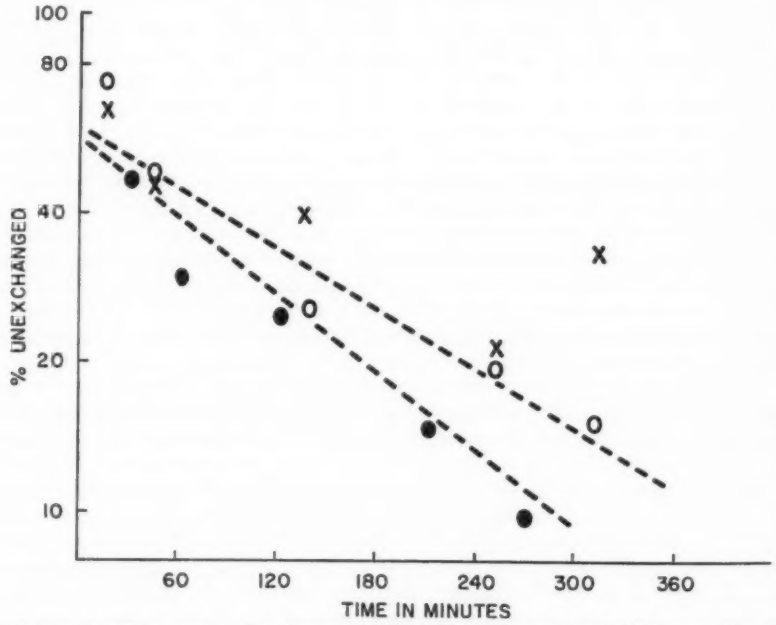

FIGURE 1. Time course of exchange of K^{42}, added to suspension fluid at zero time, with K of Mytilus sperm. Figures uncorrected for inulin space or dry weight. Dots and circles represent separate experiments; crosses are results from one run with isolated Mytilus sperm head-midpieces.

in Table III were obtained from analyses in which samples of whole sperm, head-midpiece and tail were taken from the same batches and processed during coincident time periods. Weights given are pellet weights obtained by weighing the previously tared centrifuge tubes after the supernatant fluids had been decanted, followed by inverting the tubes on filter paper for about five minutes. The gain in weight due to similar treatment of centrifuge tubes containing only pure water (or the supernatant from the centrifugation of whole sperm suspension) is in the range 10–15 milligrams (5–10% of the usual pellet weight).

With Arbacia and Mytilus sperm, head-midpieces and tail pellets contained less K and more Na and Cl than did the whole sperm, inulin spaces increasing markedly. Phascolosoma sperm heads, on the contrary, had a higher K content with lower Na and Cl, inulin spaces remaining about the same. Tail fractions of all three forms had very high inulin spaces, high Na and Cl contents and low K.

TABLE III

Relative weights, inulin spaces and Na, Cl and K concentrations of whole sperm, head-midpieces and tail pellets. Units as in Table I.

	Relative weight	Inulin space %	Na	Cl	K
Whole sperm					
Arbacia (4)	100	41	191	200	119
Mytilus (2)	100	39	244	271	44
Phascolosoma (8)	100	44	202	206	106
Heads-midpieces					
Arbacia (4)	72	56	305	329	54
Mytilus (2)	96	52	302	353	31
Phascolosoma (8)	64	44	173	192	130
Tails					
Arbacia (4)	33	85	394	401	15
Mytilus (2)	21	68	356	373	10
Phascolosoma (8)	20	92	422	384	25

Pellet weights of head and tail fractions corrected for the drainage factor (10 mg.) added together should approximate the corrected pellet weight of the whole sperm, since comparable volumes were centrifuged, starting with a standard suspension. This summation holds reasonably well for Arbacia and Phascolosoma. Mytilus sperm heads swell and hence the pellet weights of the parts add to more than the pellet weight of the whole sperm.

While head fractions undoubtedly were contaminated to a slight degree by separated tails, it is probable that the pellet weights indicate minimum values for weights of sperm tails as compared to the whole. On this basis tails make up 20–30% of the total sperm weight.

The increase in inulin space for the pellets of head fractions of Arbacia and Mytilus sperm as compared to whole sperm could represent either a general increase in inulin penetration into all units or a complete destruction of some of the units. It is impossible to select, on any rigorous basis, between these two alternatives since no method was available to us to distinguish, by independent criteria, dead heads

from live heads. However, much of the increase in Na and Cl and decrease of K could be accounted for by assuming that the increase in inulin space represented complete physiological destruction of the appropriate number of morphologically intact heads. Phascolosoma sperm would then be assumed to have tougher heads, all surviving the homogenization of the sperm suspension. Head fraction pellets of Arbacia and Mytilus sperm maintain relatively constant ionic contents over periods of several hours, thus showing that, if partial injury is responsible for the decreased K contents, the effects are not progressive. On the whole, we suggest that homogenization results in destruction of some units of the head suspension, the K content of individual intact heads remaining at at least as high a level as that of intact sperm.

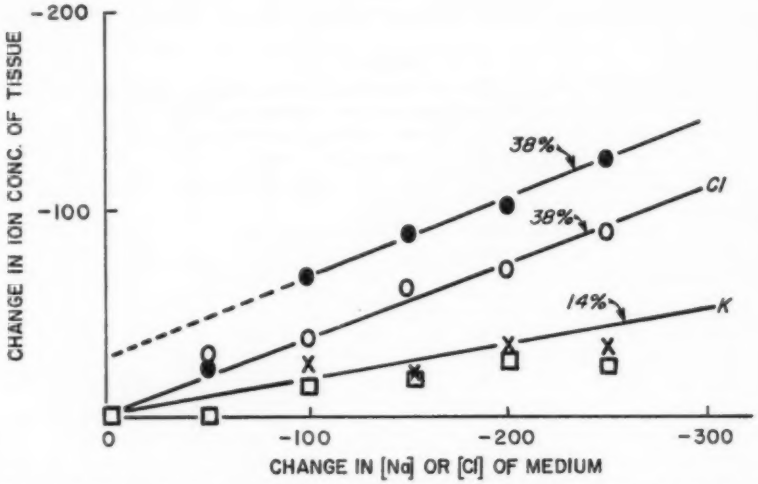

FIGURE 2. Values for Na (●), K (x), Cl (○) and pellet weights (□) of Arbacia sperm suspended 30 minutes in sea water diluted with 10 mM. KCl. Units in mM. per liter or per Kg. wet weight of pellet. The greatest dilution was a 50:50 mixture of sea water and 10 mM. KCl. Figures at arrows represent slopes of curves.

When Arbacia sperm are treated for 30 minutes with mixtures of sea water and 10 mM. KCl, thus varying Na_o, Cl_o and total ionic strength but holding K_o constant, the cells swell slightly as indicated by the increase in pellet weight (Fig. 2). In approximately 50% sea water the weight increase is slightly over 20%. Using the usual methods of calculation this would indicate a "non-osmotic" space (b value) of nearly 80%. Thus there is no evidence for an ordinary osmotic behavior of the sperm cells. As the medium is diluted, K_o holds constant, K_i decreases in somewhat greater degree than can be accounted for by the increase in cell volume. Pellet Na_i and Cl_i decrease with decrease in Na_o and Cl_o but in a manner indicating that the change, in the greater dilutions, is primarily in the inulin space fraction.

Arbacia treated with various concentrations of KCl in sea water (Na_o held constant, K_o and Cl_o varied) show a remarkably constant Na_i concentration (Table

TABLE IV

Arbacia sperm exposed to ionic concentrations indicated, prepared by adding KCl to sea water. Cell water concentrations calculated assuming 35% inulin space, 40% dry weight of sperm.

Na			$\frac{Na_i}{Na_o}$	Cl			$\frac{Cl_o}{Cl_i}$	K			$\frac{K_i}{K_o}$
Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$	
420	218	180	0.43	510	218	102	5.0	10	83	205	20.5
420	218	180	0.43	610	303	230	2.6	110	176	352	3.2
420	218	180	0.43	710	375	375	2.2	210	202	338	1.6
420	201	138	0.33	810	435	390	2.1	310	269	409	1.3
420	222	191	0.45	910	535	550	1.6	410	335	490	1.2
420	233	220	0.52	1010	650	760	1.3	510	405	580	1.1

IV). K_i and Cl_i increase markedly with no evidence of the reciprocal loss of Na_i noted for such systems as the frog sartorius. Based on calculated cell water concentrations, K_i/K_o is over 20 and Cl_o/Cl_i is 5 in normal sea water. Both ratios approach unity as total external ionic strength approaches two times that of sea water. There is no significant change in volume as total ionic strength is varied until very high concentrations are reached.

Mytilus sperm, treated in a similar fashion, show some increase in Na_i, Na_o being held constant (Table V). Both K_i and Cl_i increase somewhat but especially K_i is regulated to change much less than K_o, whereas K_i/K_o in normal sea water is about 15, the ratio is 0.5 in sea water made half molar with KCl.

In view of the interest in the use of glycerol as protective agent in freezing sperm, Mytilus sperm were suspended in 13% (by volume) of glycerol in sea water, controls being suspended in sea water diluted in corresponding fashion with distilled water. Little volume change was noted in 4–5 hours. K_i decreased with time in both glycerol-treated sperm and controls. Glycerol-treated sperm lost K and gained Na and Cl during the first half hour of treatment, the difference then being maintained. Motility in glycerol was maintained in an odd vibratile fashion, motility in the controls appeared normal throughout the experiment.

TABLE V

Mytilus sperm suspended 30 minutes in solutions of KCl in sea water as indicated. Calculations of cell H_2O concentrations assuming 40% dry weight of sperm and using inulin spaces indicated. K_i/K_o ratios calculated using cell water concentrations.

Relative volume pellet	% Inulin pellet	Na			$\frac{Na_i}{Na_o}$	Cl			$\frac{Cl_o}{Cl_i}$	K			$\frac{K_i}{K_o}$
		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$	
100	32	420	197	155	0.37	510	197	83	6.1	10	66	153	15.3
98	33	420	205	166	0.39	610	257	163	3.7	110	127	226	2.1
98	32	420	202	166	0.39	710	313	210	3.4	210	171	255	1.2
102	34	420	232	225	0.53	810	404	325	2.5	310	225	301	0.9
109	41	420	266	265	0.63	910	483	310	2.9	410	266	274	0.7
106	42	420	275	285	0.68	1010	555	375	3.1	510	310	278	0.5

Discussion

While the results reported here must be regarded as a part of a preliminary survey, certain conclusions may be drawn. In common with all cells studied, spermatozoa of the type studied maintain high K and low Na and Cl concentrations as compared to their normal active environment. In mammals this ionic differentiation becomes pronounced only after the sperm descend to the lower portions of the reproductive tract or are released. In this case the ionic gradients are probably established due to changes in the seminal fluid, not in the sperm themselves (Salisbury, 1956). The precise environments during formation and maturation of sperm of the invertebrates used in the present studies are not known to us.

As the ionic environment is altered, there is evidence that minimum portions of Na_i and K_i are held rather firmly, K_i being considerably higher than Na_i. There is no indication of a reciprocal relationship between Na_i and K_i nor is there any equality of the Donnan Ratios K_i/K_o and Cl_o/Cl_i except under very abnormal conditions (0.5 *M* KCl in sea water).

Both K_i and Na_i are rapidly and nearly completely exchanged with the environment, thus showing that ion selection does not involve a rigid sequestering of the elements. Some evidence for active extrusion mechanisms might be obtained from more precise flux measurements but no evidence for the process can be adduced from the present data. Rather, sperm behave as though they could retain relatively fixed amounts of Na and K, meanwhile allowing exchanges freely between medium and cell. In general the electrolytes of sperm behave rather similarly to those of certain smooth muscles from invertebrates (*cf.* Steinbach, 1940).

With respect to the problem of localization of the ion distribution mechanisms, these results are suggestive but by no means conclusive. It is certain that the head-midpieces of all three types of sperm retain K and continue to exclude Na and Cl with reference to the medium. With Phascolosoma, head-midpiece fractions maintain higher respective ionic gradients than do whole sperm. With Arbacia and Mytilus the gradients of head-midpiece fractions are reduced. While there is no compelling evidence, it is tentatively assumed that mechanical damage to portions of the head-midpiece fractions of Arbacia and Mytilus is responsible for the decrease in magnitude of Na_o/Na_i, K_i/K_o and Cl_o/Cl_i in the head pellets as compared to whole sperm pellets.

Tail fractions always showed very high inulin spaces and little if any ability to maintain ion gradients. It is suggested that the contractile organelle of the spermatozoan is entirely dependent on the ion-concentrating mechanisms of the head-midpiece portion for its ionic gradients, assuming such exist *in vivo*. On the basis of the known structures and metabolic activities it would be logical to look for evidence that the midpieces play major roles in the processes. Mitochondria in general are known to be able to retain selectively various ions.

Isolated sperm tails contain enzymes usually associated with contractile processes and also show spontaneous movements on application of ATP. These spontaneous movements are best described as twitchings rather than conducted waves as seen in normal attached tails. It seems possible that normal coordinated activity of sperm may depend upon ionic gradients imposed on the contractile tails by the metabolic activity of the midpiece regions.

The sperm used in these experiments were all of the simple type presumably

possessing short midpieces and tails with the 9 + 2 arrangement of fibrils (Afzelius, 1955). It is anticipated that comparative studies with mammalian and other sperm with longer midpieces and more complicated flagellar structure, indicating a possible extension of mitochondrial components, will shed further light on the compartmentalization of ionic gradients.

Summary

1. The concentrations of Na, K and Cl of pellets of sedimented sperm of Arbacia, Mytilus and Phascolosoma have been determined. With the aid of inulin space determinations on the pellets, it can be shown that sperm resemble other cells studied in maintaining, relative to the sea water environment, high internal K concentrations and low Na and Cl.

2. Sperm of the three species were separated into head-midpieces and tail fractions. The former retain the ionic selection properties of the whole sperm. Isolated tails appear freely diffusible to all constituents including inulin.

3. Using radioactive tracers it is shown that Na and K of sperm are freely exchangeable with the environment.

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SOME OBSERVATIONS ON THE GROWTH RATE OF SEA URCHINS IN THE GENUS STRONGYLOCENTROTUS[1]

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While vast amounts of basic biological information have been derived from studies using the eggs and early embryos of sea urchins, so little is known concerning the growth rate, age, and longevity of the adults that both Harvey (1956) and Comfort (1956) have remarked on the scarcity of information. Shearer, de Morgan and Fuchs (1914), Elmhirst (1922), Aiyar (1935), Moore (1935), Bull (1938), Nataf (1954), and Lewis (1958) have contributed important information for species in a number of different genera. Despite the fact that the green sea urchin *Strongylocentrotus droebachiensis* (O. F. Müller) occurs in suitable habitats throughout much of the northern half of the northern hemisphere and in places is one of the most conspicuous and numerous macroscopic members of the marine benthos and intertidal fauna, data on its growth rate are few. Soot-Ryen (1924) cautiously presented a few data based on relatively small collections from Ramfjorde on the Norwegian coast and clearly indicated his doubt as to the justification of defining year-classes. He also pointed out that (p. 16) "*Var. pallida*, G. O. Sars, lebt mit *f. typica* zusammen." Thus, in view of Vasseur's (1951, 1952) findings, it would appear that Soot-Ryen was dealing with a collection including two species. Schorygin (1928) presented size-frequency distributions based on large collections from the Barents Sea, but his separating *S. droebachiensis var. atroviolaceus* from what he considered the typical form of the species suggests that his data were derived from the study of *S. pallidus* (G. O. Sars). Grieg (1928) presented data on size-frequency distributions for both the Folden Fjord and for Bals Fjord but made no mention of the existence of more than one form or species in the genus. Some of his collections were dredged from depths as great as 200 meters, and Vasseur (1951, 1952) found *S. pallidus* to be the commoner species along the Norwegian coast in deeper dredgings. Thus it appears likely that he was dealing with both species without distinguishing between them. The present writer (Swan, 1958) has reported the size-frequency distribution of a collection of *S. droebachiensis* made intertidally in York, Maine. There appears to be no doubt that these belong to the species considered by Vasseur (1951) as *S. droebachiensis*, but with the existence of two species of *Strongylocentrotus* at Dröbak (Vasseur, 1952) Mortensen's (1943, p. 214) statement concerning Müller's type to the effect that "the fact that it is polyporous is a definite proof of the correctness of identifying the common N. Atlantic species with it, this being the only polyporous Echinoid of the N. Atlantic region" becomes a weak argument. Madsen's (1959) figure and discus-

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sion of this type specimen make it appear likely that determining its identity with either species would be nearly if not quite impossible.

On the west coast of the United States *S. purpuratus* (Stimpson) is probably the sea urchin most used for embryological, physiological, and biochemical studies, and in Japan other members of the genus and the closely related *Hemicentrotus pulcherrimus* (A. Agassiz) are extensively used for these purposes. To the writer's knowledge there are no available data pertaining to the growth rates of any of these species. It is the purpose of the present work to begin to fill these gaps.

Experiments and Observations at New Castle, New Hampshire, and York, Maine

On the morning low tides of June 1 and 3, 1958, sea urchins (*S. droebachiensis*) were collected from near the southeast corner of the island on which Nubble Light off Cape Neddick is located (at 43° 09′ 04″N and 70° 35′31″W in the township of York, Maine). Except for the time they were actually being measured, the animals were kept refrigerated until evening of the same day, when they were placed in cages (Swan, 1960) submerged in a large tide pool (about 140 × 60 feet with a surface water level about one foot above mean low water and a maximum depth of about three feet at tide levels below that of the pool) at New Castle, N. H. (43° 3-2/3′N, 70°42-4/5′W). The place of collection was the locality studied earlier (Swan, 1958). Only those specimens approximating 8–10, 24–26, 40–42, or 50–52 mm. in test diameter were collected. Between their collection and their deposit in the cages, each specimen was re-measured, and those not within the desired size ranges were discarded. Three cages were used, and the animals of one of the three larger size groups were placed in each. The 8–10 mm. individuals were divided among four one-pint polyethylene containers, perforated with several holes (3–5 mm. in diameter) through the cover and bottom. Then the containers were pressed into holes in the cross-pieces of the cages.

The tide pool naturally supports a moderate population of sea urchins; is well protected from heavy surf by ledges; is near kelp beds, which furnished food for the caged animals; and is behind a private estate and thus free from much interference from the public. The cages were securely anchored to the bottom of the pool where its depth at low water was about 1½ feet. When the animals were placed in them, several nearly entire healthy plants of the kelp *Laminaria digitata* (Lamouroux) and/or similar-appearing species were added to each cage, and pieces of the blades were placed in the plastic boxes in such manner that they would not obstruct the passage of water. Subsequently, until the experiments were terminated on June 3, 1959, the cages were visited and the kelp was renewed at intervals ranging from 6 to 18 days. When the experiment was ended, the animals were killed in formalin in sea water and after 24 hours dried at room temperature. Their spines were brushed off, and the tests were measured with vernier calipers. The results of this experiment are summarized in Table I.

The almost perfect viability is notable. The single specimen lost from the group of greatest diameter died within the first month. Two others were accidentally crushed. In previous experiments of this type there was some evidence of disease as a possible cause of mortality. It appears to be important to observe the following practices: (1) keeping the animals in good condition between the time

of their collection and their return to the ocean, (2) feeding only fresh living kelp, and (3) keeping the cages clean of anything that might impair the free passage of water through them.

The sizes of animals at the beginning of the experiment were selected largely on the basis of size-frequency modes found or suspected in the earlier study (Swan, 1958).

One of the factors that might be expected to affect growth rate is the material utilized as food. Earlier experiments confirm this. On June 12 and 13, 1956, 129 of the smallest individuals that could be found were collected from the Nubble. These were divided into five groups having approximately the same distribution of sizes. One group was immediately preserved and dried. The other four groups were put in one-pint polyethylene containers, two of which were supplied with *Laminaria* and two with *Ascophyllum nodosum* (L.) Le Jolis. These were all placed in a cage in the tide pool at New Castle. On June 26 one of the containers with each alga and its urchins was taken to Boothbay Harbor, Maine, where

TABLE I

Data on the growth of Strongylocentrotus droebachiensis *(O. F. Müller) held in cages at New Castle, N. H. from June 1 or 3, 1958, to June 3, 1959*

Original		Concluding			
Test diam. (mm.)	#	#	Test diameters (mm.)		
			Range	Mean	Standard deviation
8–10	41	40	23.1–31.6	27.5	±2.2
24–26	42	41	33.4–45.3	40.2	±2.8
40–42	39	38	45.3–54.1	49.8	±2.1
50–52	16	15	51.4–57.3	55.5	±1.7

through the courtesy of the U. S. Fish and Wildlife Service and the Maine Dept. of Sea and Shore Fisheries the writer was able to suspend them in a cage from their common dock. Before the end of the first month mortality had reduced each of the groups at Boothbay Harbor to 20 specimens and those at New Castle to 18 with the *Ascophyllum* and 17 with the *Laminaria*. On August 23, the living specimens at Boothbay Harbor were measured as were those at New Castle two days later. Those fed *Ascophyllum* at Boothbay Harbor averaged 6.9 mm. in diameter whereas those at New Castle averaged 7.4 mm. With *Laminaria* those at Boothbay Harbor averaged 8.2 mm. and those at New Castle 8.9 mm. For numerous reasons these figures and the implied amounts of growth cannot be considered accurate, but they do suggest that there is probably a difference in growth rates between localities even when the supplied food is the same. On September 13 all the animals at Boothbay Harbor were found dead, apparently as a result of plugging of the holes in the polyethylene containers.

On June 11, 1957, nearly a year after the initiation of this experiment, 16 animals with each kind of food were still living at New Castle. These were preserved and dried. The dried specimens are shown in Figure 1, and the data obtained are given

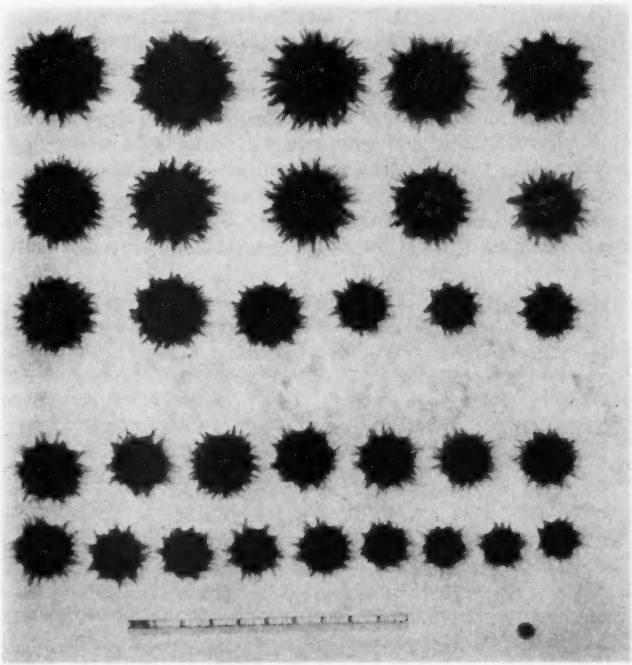

FIGURE 1. Effect of differences in food over a period of one year on the growth rate of *S. droebachiensis* (O. F. Müller) held in plastic containers at New Castle, N. H. Upper three rows, fed *Laminaria*. Lower two rows, fed *Ascophyllum*. Lower right, specimen as originally collected. (Photograph by George M. Moore.) Rule length = 10 cm.

in Table II. From these figures it appears that the increase in mean diameter of of the specimens fed *Laminaria* exceeded that of those fed *Ascophyllum* by more than 70%. This should not be interpreted as meaning that *Laminaria* is more nutritious. While no quantitative study was made, it appeared that much more of the *Laminaria* was eaten.

TABLE II

Data on growth of Strongylocentrotus droebachiensis *supplied with two different algae and held in cages at New Castle, N. H. from June 12 or 13, 1956, to June 11, 1957*

	Numbers		Diameters (mm.)		
	Original	Preserved	Range	Mean	σ
Basic controls preserved immediately	25	25	3.5–6.1	5.0	±0.63
Those fed *Ascophyllum*	27	16	11.8–18.6	15.4	±2.07
Those fed *Laminaria*	27	16	14.7–27.7	22.1	±4.08

On June 11 and 12, 1957, another series of experiments was begun, but by November 4, mortality was so high that these were terminated. The following data were recorded. (1) 16 of 17 small *S. droebachiensis* (initially 7–10 mm.) lived in perforated polyethylene containers with no food added for four months. After that many died, but so did many others supplied with various algae and kept nearby. (2) Small *S. droebachiensis* eat and grow well when supplied *Corallina officinalis* L. or *Chondrus crispus* (L.) Stackhouse, but these tend to break or to be chewed so that after a few days there are many small pieces in the containers. These pieces tend to clog the holes and thus impede water circulation. For feeding larger animals in the cages, much of either of these algae was soon lost through the wire mesh.

TABLE III

Data on growth of Strongylocentrotus *held in cages at Friday Harbor, Washington, in 1959 and 1960*

Start of experiment				Conclusion of experiment			
Date 1959	#	Diameters (mm.)		Date 1960	#	Diameters (mm.)	
		Range	Mean			Range	Mean
S. droebachiensis							
July 6	12	26–32	ca. 30	July 5	11	47.2–59.7	52.8
Aug. 12	10	55–57	ca. 56	Aug. 8	9	57.8–66.6	62.1
Aug. 11	4	76–78	ca. 76½	Aug. 8	1	77.2	—
S. echinoides							
July 26	17	25–27	ca. 26	July 26	15	36.3–44.3	40.6
July 26	20	41–43	ca. 42	July 26	14	44.5–53.6	48.6
July 26	8	53–57	ca. 55	July 26	7	54.1–60.7	57.1
S. franciscanus							
July 6	15	26½–31	ca. 29	July 5	15	45.0–59.3	54.2
July 6	15	46½–52½	ca. 49	July 5	14	56.9–67.2	62.0
S. purpuratus							
July 8	2	11 & 19	15.0	July 5	2	23.4–39.9	31.7
July 16–20	12	24–28	ca. 26	July 18	10	37.0–48.2	42.6

EXPERIMENTS AND OBSERVATIONS AT FRIDAY HARBOR, WASHINGTON

While the writer was at the Friday Harbor Laboratories of the University of Washington, numerous courtesies were extended to him by Dr. R. L. Fernald, the Director of the Laboratories, and other members of the staff. Thus he was able to collect an assortment of sea urchins belonging to the genus *Strongylocentrotus* and to suspend some of these in cages from the Laboratories' dock for observations on growth rate. All urchins caged for study at Friday Harbor were fed the large kelp, *Nereocystis luetkeana* (Mertens) Postels and Ruprecht. A summary of the data so obtained is given in Table III.

From the data obtained on *S. droebachiensis,* even though they are rather small in amount, it appears quite obvious that at Friday Harbor with a diet of *Nereo-*

cystis this species grows both more rapidly and to a larger size than it does at New Castle, N. H., with a diet of *Laminaria*. Whether this is a result of differences in the food, differences in other environmental factors, genetic differences between the populations, or to some combination of these remains unanswered.

S. echinoides A. Agassiz and H. L. Clark (which, as suggested by Vasseur (1952), is closely allied to or possibly even identical with *S. pallidus*) grew at a decidedly different and slower rate for animals of comparable sizes.

Limitations in cage space prevented studies with the long-spined and often very large *S. franciscanus* (A. Agassiz) except in small sizes. The data obtained from these latter suggest that this species in its smaller sizes grows a little more rapidly than does *S. droebachiensis* under comparable conditions. This suggests that the large size often attained by individuals of the species is largely the result of continuing growth rather than from more rapid early growth.

The few data obtained for *S. purpuratus* (Stimpson) indicate a relatively slow growth rate, comparable with that of *S. echinoides* in the smaller sizes; but the large sizes attained by the former species in this region (Swan, 1953) indicate that its growth must continue with less reduction in rate for a longer time.

Discussion

On June 29, 1953, a considerable number of sea urchins ranging from about ½ to 1½ mm. in test diameter were collected at the "Nubble." These appear to have been the young of the year. When the collection was made at the same place four years later (Swan, 1958) no such small animals were found, but well-defined modes in the size-frequency distribution were found at 8–10 and 24–26 mm., and possible modes 40–42 and 52–54 mm. Thus when the 8–10 mm. animals in the present experiments grew to a mean diameter of 27.5 mm. in a year, we have close agreement with the 24–26 mm. diameter for the next year class. Likewise the growth of the 24–26 mm. animals to a mean diameter of 40.1 mm. confirms the 40–42 mm. mode for the next year class. The fact that the 40–42 mm. animals attained a mean diameter of only 49.8 mm. in the year may suggest that the previously suspected mode at 52–54 mm. is too high, and a re-examination of the earlier size-frequency distribution (Swan, 1958) suggests the possibility of a mode between 40–42 and 52–54, possibly at about 46 mm. However, at these sizes and ages overlaps in age groups become great and numbers of individuals small. Thus extreme care in interpretation is required. That the group initially 50–52 mm. in diameter attained a mean diameter of 55.5 mm. is perhaps notable, as is the near lack of mortality in this group. Only three of the 1305 individuals studied earlier exceeded 54 mm. in diameter. Of the factors that could account for this continued life and growth the following should be considered: (1) the caged animals were not subject to the collection for food by man; (2) the cages may have afforded protection against other predators and against some physical environmental factors (as wave action and moving stones); (3) *Laminaria* for food was always available; and (4) there may be unrecognized differences between conditions at the "Nubble" and those of the tidepool that affect growth. In this latter respect surf action may affect available feeding time. At the "Nubble" the urchins may be severely beaten by waves as often as four times a day, whereas at the tide pool surf action is very slight.

The growth of these largest individuals suggests three more questions concerning

the life of *Strongylocentrotus droebachiensis* in these localities: (1) What is the longevity of the animals? (2) Do they grow as long as they live? (3) Do they reproduce as long as they live?

For comparison of these findings with those of earlier works on *"S. droebachiensis,"* estimated diameters at various ages are listed in Table IV. The exact time in the year when the collections were made varies some, but all were from essentially summer work. In the different localities there may be differences in time of spawning and in the settling of the larvae; and as mentioned previously, the data of the earlier workers may well represent collections of *S. pallidus* or mixtures of it with *S. droebachiensis*. To take the estimates made on dredged material as proof of age at various sizes is going beyond justification, but a few observations may be worth noting. It appears probable that Soot-Ryen

TABLE IV

Sizes of "S. droebachiensis" *as estimated at various ages in millimeters of test diameter (Numbers without parentheses indicate size range in class; numbers in parentheses indicate estimate of modal size for the class)*

Age class	Swan N. H. & Me., U. S. A.	Soot-Ryen (1924) Ramfjorde, Norway	Schorygin (1928) Barents Sea	Grieg (1928) Folden and Bals Fjords, Norway
0	½–1½	6–10	—	½–2.5
I	(8–10)	12–22 (18)	12–20	(5–6)
II	(24–26)	22–33 (28)	21–31	(15)
III	(40–42)	33–40 (37–38)	32–41	(24 & 32)
IV	(46–54?)	43–52 (48)	42–52	(40?)
V		52–60	53–60	(50?)

missed the class of the year and that his groups are listed a year too young. It also appears possible that Schorygin's estimates are displaced by a year and that his 12–20 mm. group is actually the II-year class. If these speculations are correct, the figures of the earlier workers are in amazingly close agreement with each other, and it took the animals they studied about five years to grow to the size reached by the animals studied on the northern New England coast in four.

The data obtained at Friday Harbor are so few that further comments are scarcely justified. It is quite obvious that in the smaller sizes the *S. droebachiensis* grown in cages there grew much more rapidly than in New Hampshire. In fact specimens grew from a size scarcely larger than those on the New Hampshire coast would be at two years to their size at four or more years in a single year. As mentioned earlier the factors involved in this rapid growth have not been determined. While it may be pure coincidence, the growth attained by the experimental *S. echinoides* in Friday Harbor almost exactly agrees with the earlier worker's findings for the growth rate of their *"S. droebachiensis"* on the northern coasts of Europe. Since *S. echinoides* is very close to *S. pallidus*, with which species they may have been dealing, at least in part, this observation may be significant.

SUMMARY

1. For certain areas of the New Hampshire and southern Maine coasts, evidence has been accumulating indicating that in June of the year of settling *S. droebachi-*

ensis have attained a size of only ½–1½ mm. in test diameter, and that the modes of subsequent year classes are approximately 8–10, 24–26, 40–42 and somewhere between 46 and 54 mm.

2. *S. droebachiensis* was found to grow considerably faster in test cages at Friday Harbor, Washington, but differences in the food supplied might be responsible, at least in part, for this difference.

3. Exploratory tests on growth rate were carried out at Friday Harbor on *S. echinoides, S. purpuratus,* and *S. franciscanus.*

4. Observations of earlier workers on growth rate in sea urchins of the genus *Strongylocentrotus* have been reviewed and compared with the present findings.

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